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SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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VARIANTS OF HEMOLYTIC STREPTOCOCCI; THEIR RELATION TO TYPE-SPECIFIC SUBSTANCE, VIRULENCE, AND TOXIN.

By E. W. TODD,* M.D., and R. C. LANCEFIELD, Ph.D

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(Received for publication, August 7, 1928.)

In previous communications one of us (1) described three substances which can be extracted from hemolytic streptococci: (1) *The nucleoprotein P* is common to all strains of hemolytic streptococci and is serologically related to the nucleoproteins of pneumococci and of green streptococci. (2) *The non-protein substance C*, which appears to be a carbohydrate, is found in all strains of hemolytic streptococci but is species-specific and serologically distinct from the carbohydrate fractions of pneumococci and green streptococci. (3) *The type-specific fraction M*, which is probably protein in nature, has not been isolated from other species of microorganisms; it occurs in serologically distinguishable forms which serve to differentiate hemolytic streptococci into types.

One of us (2) has previously described two forms of hemolytic streptococci distinguishable by the morphology of their colonies. The general appearances of these colonies, when grown on a special medium and viewed by reflected light, are the same as those which distinguish the rough and smooth varieties of other bacteria but the terms "R" and "S" have not been used and the colonies have been designated "matt" and "glossy" to avoid confusion which would otherwise certainly arise from the circumstance that the rough, or matt, colonies are the virulent type while the smooth, or glossy, forms are relatively avirulent. It is the purpose of this paper to show that the type-specific substance M is present in the potentially virulent organisms comprising the matt variety of colony and that it is not present in the avirulent variant cocci which form glossy colonies.

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It will also be shown that filtrates from both matt and glossy cultures of hemolytic streptococci contain skin-reactive toxin.

Some Characteristics of the Type-Specific Substance M.

The type-specific substance M is prepared by a modification of Porges' (3) method. The bacterial bodies are extracted with N/20 HCl in salt solution at the temperature of boiling water; and after neutralization and clarification by centrifuging the resulting clear slightly yellow fluid is used as an antigen for precipitin reactions. Bacterial extracts prepared in this manner contain the type-specific substance M and in addition they also contain small quantities of the non-type-specific fractions, P and C, which may cause some precipitation with sera prepared against any strain of hemolytic streptococcus. To avoid the appearance of these confusing precipitates the serum may be absorbed with any heterologous strain of hemolytic streptococcus and by this means a specific anti-M serum is obtained which will only precipitate in the presence of the homologous M substance.

In the original work (1) which led to the recognition of the type-specific substance M thirteen strains were used which Dochez, Avery and Lancefield (4) had classified, some years earlier, into types by agglutination and protection tests. Ten of these strains yielded type-specific precipitating substances which differentiated them into types corresponding to those originally determined. The remaining three strains failed to yield any type-specific substance although they had been classified by agglutination and protection in the earlier work. It was suggested that prolonged cultivation in the laboratory had caused these three strains to lose their type-specific characteristics. In the present communication it will be shown that matt cultures containing the type-specific substance can, by various means, be reduced to the glossy form in which the type-specific substance is no longer present.

The Preparation of Anti-M Serum.

As the type-specific substance M, after separation from the bacterial cell, does not produce any demonstrable antibody, when injected into animals, the only antigen available for preparing anti-M serum

is a suspension of bacteria in the matt form. Matt cocci contain the three substances P, C and M and sera prepared with these organisms, therefore, contain antibodies to each of the three substances. On the other hand, cocci in the glossy form contain the two substances P and C but are devoid of the type-specific substance M; consequently, antibacterial sera prepared with glossy strains contain antibodies to P and C but do not contain any type-specific antibody.

Anti-M sera were prepared by inoculating rabbits intravenously with 16 hour cultures of matt cocci grown in tryptic broth. Immunization was commenced with four injections on consecutive days of 1 cc. of heat-killed culture, followed a week later by four doses of 2 cc. of the same vaccine. During the 3rd week the rabbits received four doses of 0.5 cc. of living culture and in the 4th week this dose was doubled. A final series of doses of 2 cc. of living culture was given during the 5th week and the animals were bled 10 days after the last injection. The sera of animals immunized in this way usually contained a satisfactory quantity of antibody to the type-specific substance M but with some strains it was necessary to continue immunization with 5 cc. and even 10 cc. of living culture before useful sera could be secured. A few strains have been encountered which, although they were in the matt form and moderately virulent for mice (0.001 cc. or 0.0001 cc.), produced only traces of type-specific antibody in rabbits even after intensive immunization. Twelve rats were immunized with a strain (New York V E14) which had previously failed to produce more than traces of type-specific antibody in the sera of twelve immunized rabbits. The rats, which remained perfectly well during immunization, received the following intraperitoneal doses: 1st week 3 doses of 0.25 cc. of heat-killed culture; 2nd week 4 doses of 0.5 cc. of heat-killed culture; 3rd week 4 doses of 1.0 cc. of heat-killed culture; 4th week 3 doses of 1.0 cc. of living culture; 5th week 4 doses of 2.0 cc. of living culture; 6th week 4 doses of 2.0 cc. of living culture. Seven of the immunized rats yielded moderately good anti-M sera; the remaining five sera contained traces of type-specific antibody. As rats appear to be able to tolerate relatively larger doses of culture than rabbits it is possible that they may be more suitable for the preparation of anti-M serum; but the small yield of serum makes this method impracticable for routine purposes.

The Absence of the Type-Specific Substance M from Organisms Which Form Glossy Colonies.

It has already been stated that the type-specific substance M is found in HCl extracts of matt hemolytic streptococci and that it is not found in similar extracts prepared from the glossy variants.

This is demonstrated by the following experiment:

Four type-specific anti-M sera were prepared by immunizing rabbits with four matt strains of hemolytic streptococci belonging to different serological types and by subsequently removing the non-type-specific antibodies from the sera by absorption with heterologous strains.

Table I gives the precipitin reactions of the four sera with HCl extracts prepared from cultures of the homologous cocci (1) in the matt form and (2) in the glossy variant form.

TABLE I.

Precipitin Reactions of Type-Specific Anti-M Sera with Extracts of the Homologous Strains (1) in the Matt Form, (2) in the Glossy Form.

Volumes of extracts*	Strain S43		Strain S23		Strain C203		Strain London	
	1 Extract from matt form	2 Extract from glossy form	1 Extract from matt form	2 Extract from glossy form	1 Extract from matt form	2 Extract from glossy form	1 Extract from matt form	2 Extract from glossy form
cc.								
0.4	+++	−	++++	−	++	±	++	−
0.1	++	−	++	−	++±	−	++±	−
0.025	+	−	+	−	++	−	+±	−

* These volumes were made up to 0.4 cc. with saline, and 0.1 cc. of serum was added to each tube.

It will be seen from Table I that the extract prepared from the matt form of each strain gave a good precipitin reaction with the homologous antiserum; on the other hand, the extract prepared from the glossy form of each strain gave a negative precipitin reaction with the single exception of Strain C203 which gave a faintly positive reaction. Although this experiment seems to show that each of the four strains lost its type-specific substance in the process of degradation to the glossy variant form, yet it will be seen from the results of experiments with highly concentrated extracts that, in reality, only one strain had completely lost its type-specific substance. Highly concentrated extracts were prepared from each of the four glossy variants referred to in Table I in the following manner: The centrifuged deposit from 9 liters of broth culture was extracted with HCl; and, after concentration by alcoholic precipitation, the precipitate was redissolved in

5 cc. of salt solution. Precipitin tests, with the concentrated extract prepared from Strain S23 were negative showing that this strain was completely devoid of type-specific substance. Precipitin tests, with the other three concentrated extracts and their homologous specific anti-M sera, were weakly positive showing that three of the glossy cultures retained traces of type-specific substance. The minute amounts of specific substance remaining in these cultures can be judged from the following figures—9 liters of broth culture were used in preparing extracts from the glossy forms—50 cc. of broth culture were used in preparing extracts from the matt forms—180 times more culture was, therefore, used in the preparation of the glossy extracts than in the preparation of the matt extracts and, in spite of these disproportionate quantities, the latter extracts contained the larger quantity of the type-specific substance M. It appears from these experiments that hemolytic streptococci are rarely degraded to the point at which type-specific substance completely disappears.

Some Characteristics of Matt Cultures of Hemolytic Streptococci.

Twenty-eight strains of hemolytic streptococci were examined immediately after isolation from pathological conditions in the human body. The sources of these cultures included cases of puerperal septicemia, pleural effusion, scarlet fever, pneumonia and sinusitis; strains were also isolated from the depths of enucleated tonsils and from throat swabs. In twenty-one cases the cultures when freshly isolated, were entirely composed of matt colonies; in five cases both matt and glossy colonies were seen on the plates; and in two cases the cultures were entirely glossy, but as both the glossy strains were obtained from throat swabs and were accompanied by other bacteria there was no evidence that the hemolytic streptococci were playing a pathogenic rôle. Table II gives the source and character of the cultures.

There is, therefore, some evidence that cultures freshly isolated from human sources are usually of the matt variety and this statement particularly applies to diseases such as septicemia in which the streptococci are the undoubted causal agent.

It is frequently found that matt strains of hemolytic streptococci, isolated from human lesions and undoubtedly pathogenic for man, are

TABLE II.

The Morphological Appearance of Colonies of Freshly Isolated Strains of Hemolytic Streptococci and the Source of the Cultures.

No.	Disease	Source of culture	Pure culture or mixed flora	Morphology of colonies
1	Puerperal septicemia	Blood culture	Pure culture	Matt
2	" "	" "	" "	"
3	" "	" "	" "	"
4	Nephritis and septicemia	" "	" "	"
5	Endocarditis	Postmortem culture from spleen	" "	"
6	Pneumonia and pleural effusion	Chest fluid	" "	"
7	Sinusitis	Nasal mucus	" "	"
8	Scarlet fever	Throat swab	Almost pure culture	"
9	" "	" "	" " "	"
10	Tonsillitis	" "	" " "	"
11	Enlarged tonsils	From depths of enucleated tonsils	" " "	"
12	" "	" "	" " "	"
13	" "	" "	" " "	"
14	" "	" "	" " "	"
15	Pharyngitis	Swab from pharynx	" " "	"
16	Sinusitis	" " nose	" " "	"
17	Tonsillitis	" " tonsils	" " "	"
18	"	" " "	" " "	"
19	Pneumonia	Sputum	Chiefly *S. hæmolyticus; B. influenzæ*	"
20	" (Type IV pneumococcus)	"	Mixed flora	"
21	" " " "	"	Equal numbers hemolytic streptococci and pneumococci	"

22	Pneumonia (Type IV pneumococcus)	Sputum	Mixed flora	Matt and glossy
23	Enlarged tonsils	From depths of enucleated tonsils	Pure culture	" " "
24	" "	" "	Almost pure culture	" " "
25	" "	" "	" " "	" " "
26	" "	" "	" " "	" " "
27	Tonsillitis	Swab from tonsils	Mixed flora, about 5 per cent hemolytic streptococci	Glossy
28	Pneumonia (Type IV pneumococcus)	" " "	Mixed flora	"

avirulent for mice (M.L.D. 0.5 cc. or 1.0 cc.). Such a culture entirely composed of matt colonies will be referred to in this paper as the matt attenuated form because it is avirulent for mice yet possesses the colony characteristics and the specific substance of the virulent form. Attempts to increase the virulence of matt attenuated cultures, by mouse passage, have always been successful although some of the strains tested have required very many passages before the maximal virulence of 0.000001 cc. has been attained and in some cases the virulence has never risen above 0.0001 cc. even after 80 or 90 consecutive passages through mice.

Virulence is the only quality which distinguishes the matt virulent form from the matt attenuated variety as these colonies are identical in appearance, and serological examination of HCl extracts does not show any significant difference in the quantity of type-specific substance which can be extracted from equal volumes of the two cultures. From these experiments it appears that the matt form, which is always potentially virulent, may occur in, at least, two separate varieties characterized by quantitative differences in virulence for mice; and it is probable that, by suitable passage experiments, additional forms can be obtained distinguishable by different degrees of virulence for other species of animals.

Methods of Converting the Matt Form to the Glossy Variant.

The degree of ease with which glossy variants can be obtained from different strains varies enormously. In some cases great difficulty is experienced in maintaining laboratory stock cultures of the matt, or potentially virulent, form as they spontaneously change to the glossy variant even when stored in blood broth in the ice box. In these cases it is necessary to resort to frequent mouse passages to prevent the total loss of the matt form. On the other hand, matt strains have been encountered which do not show any tendency to change to the glossy variant after repeated subcultivations on agar. Intermediate between these extremes are strains which develop a small proportion of glossy colonies after repeated subcultivations on agar. When one of the glossy colonies derived from these strains is subcultured in broth and replated on agar a mixture of matt and glossy colonies usually appears but occasionally a pure glossy culture

may be obtained by this method. By repeated selection of glossy colonies and subcultivation in broth a culture composed entirely of glossy colonies can often be obtained and in some instances a pure culture of the glossy variant can be secured by the simple process of repeated subcultivation on agar slants.

Griffith (5) and others (6–8) have shown that smooth pneumococci can be converted to the rough form by cultivation in the homologous anti-S serum. We have applied this technique to hemolytic streptococci and have found that the cultivation of matt strains in undiluted homologous anti-M serum of high titer is the quickest and most reliable method of obtaining glossy variants, and with some highly stable matt strains this is the only method by which we have been able to secure the glossy form. Here again there are wide differences between individual strains; in some cases, a few transfers in serum suffice to convert a virulent matt culture, containing abundant M substance, into the glossy avirulent variant devoid of any specific substance; in other cases, after as many as 90 transfers in high titer serum traces of the type-specific substance M can still be detected in concentrated bacterial extracts, although the colonies appear to be glossy and the organisms have lost their virulence for mice. Attempts have been made to rid these cultures of the remaining traces of type-specific substance by alternately cultivating the cocci in immune serum, plating out, selecting the most glossy colonies and again subculturing in immune serum; one strain was subjected to this treatment thirty times after 90 previous consecutive transfers in immune serum but at the end of the experiment it still retained traces of the specific substance.

Some Characteristics of Glossy Cultures of Hemolytic Streptococci.

The glossy variant is avirulent for mice in comparison with the matt virulent culture from which it is derived and attempts to raise the virulence of the variants by mouse passage have usually been unsuccessful. It is, however, possible to obtain glossy cultures which are partially virulent for mice. A strain which, in the matt virulent form, killed mice regularly in doses of 0.000001 cc. or 0.0000001 cc. was cultivated in the homologous anti-M serum and, after 55 transfers in 50 per cent serum, a pure culture of the glossy variant was ob-

tained which did not contain any type-specific substance, yet this glossy culture was sufficiently virulent to kill mice regularly in doses of 0.01 cc. and occasionally in doses of 0.001 cc. More prolonged cultivation in anti-M serum did not cause any further decrease in virulence. The partially virulent glossy culture was passed through twenty-five mice intraperitoneally but the virulence remained unchanged and there was no reappearance of type-specific substance. This appears to be an exceptional strain as the M.L.D. of the majority of glossy strains is 0.5 cc. or 1.0 cc.

During the process of conversion from matt to glossy various types of colonies appear, which may possibly represent intermediate forms or may be due to individual colonies containing a mixture of matt and glossy cocci. We have observed that different strains sometimes show peculiarities in the morphology of their colonies which are so striking that the strain can be recognized either in the matt or glossy form. In addition to these strain peculiarities other varieties of colonies appear during the gradual change from matt to glossy. Griffith (9) has noted that in spite of the apparently diverse appearances of streptococcal colonies three forms can generally be distinguished. Two of his forms appear to correspond to our matt and glossy colonies and the third is characterized by a soft consistency, a whitish opaque raised center and a thin translucent margin. In a previous communication one of us (10) described a similar form of colony, which differed in the important respect of being tough instead of watery; but further observation has shown that colonies characterized by a flat marginal zone surrounding a central eminence may occur in two forms, corresponding to the matt or to the glossy state. The matt form of this colony is opaque and of tough consistency with a central eminence surrounded by a flat marginal zone; the glossy form has a similar contour but is soft and watery. The irregular shape of these colonies causes difficulty in observing the light-reflecting character of their surfaces but the matt and glossy forms can generally be distinguished by other characteristics. These observations seem to indicate that this third type of colony is not a distinct entity separate from the matt and glossy forms; but we have failed to determine the significance of these very characteristic colonies.

The classification of colonies is further complicated by the occasional appearance of pseudoglossy forms. When a matt culture is spread on a plate the colonies in close proximity to each other may present the typical matt appearance but widely separated colonies in the same culture may be glossy in appearance. If one of the latter colonies is selected and spread on a fresh plate a pure culture of typical matt colonies may result. Pseudoglossy colonies are generally larger than true matt or true glossy colonies but they so nearly resemble the true glossy form that they are liable to cause confusion.

Owing to these variations in the appearance of colonies we have been unable to rely entirely on the colony form as a guide to the character of cultures. The criteria we have used to determine when a culture is completely degraded from the matt state are: (1) that concentrated HCl extracts of glossy cultures should not cause any precipitation when mixed with pure homologous anti-M serum (absorbed with a heterologous strain to remove the antibodies to P and C); (2) that the result of the above test should remain unchanged after the culture has been passed through a mouse.

It will be seen in the detailed description of experiments that we have only been able to secure one strain in this completely degraded state.

Reversion of Glossy Cultures to the Matt Form.

Dawson and Avery (11) have shown that many strains of R pneumococci can be reverted to the S form by repeated mouse passages or by cultivation *in vitro* in anti-R serum. Griffith (12) has shown that R pneumococci frequently revert when they are mixed with large doses of heat-killed S pneumococci and inoculated subcutaneously into mice.

We have attempted to revert glossy hemolytic streptococci to the matt form by each of these three methods.

1. Mouse Passage.—Passage experiments have been done with glossy cultures derived from five different strains (S3, S23, Henson, S43, C203) but no definite evidence has been obtained that reversion can be achieved by this method. In two cases, S3 (ten passages) and Henson (twenty-five passages), the glossy character of the cultures was judged entirely by the appearance of the colonies as no anti-M serum was available for these strains.

The virulence (0.1 cc.) and colony form of Strain S3 remained unchanged after passage through ten mice.

Passage of Strain Henson through twenty-five mice caused the virulence of the culture to rise from 0.5 cc. to 0.01 cc. and this change was accompanied by a slight alteration in colony form, many of the colonies in the passage culture having flat tops instead of the typical dome-shaped appearance of glossy colonies. In this instance the partial restoration of virulence and the accompanying change in colony structure may possibly indicate that reversion had commenced but the evidence is inconclusive in the absence of serological proof that the culture had been completely degraded before the mouse passages were commenced.

The glossy culture of Strain S23 appeared to be completely degraded as no trace of precipitate was formed when highly concentrated HCl extracts were mixed with the homologous type-specific antibody. This culture was passed through twenty-seven mice and at the end of the experiment the virulence for mice (0.01 cc.) and the colony form remained unchanged and there was no reappearance of type-specific substance.

The glossy culture of Strain S43 was not completely degraded as, although unconcentrated extracts of the variant culture did not precipitate the homologous anti-M serum, yet traces of type-specific substance could be demonstrated in concentrated HCl extracts. This culture was passed through a series of mice and examination of unconcentrated extracts, after each passage, showed the gradual reappearance of type-specific substance so that after eight passages the culture was equal to the original matt form in its yield of type-specific substance. The virulence (0.1 cc.) and colony form were unaltered by eight passages but this experiment seems to indicate that a culture which has lost the major part of its type-specific substance and yet retained a fraction of its original specificity can be reverted to the original form with comparative ease.

The variant culture of Strain C203 formed typical glossy colonies but concentrated HCl extracts of the cocci contained traces of type-specific substance. After passage through ten mice the virulence (0.1 cc.) and the colony form of the culture remained unchanged and there was no increase in the quantity of type-specific substance which could be extracted from the cocci.

2. Cultivation in Immune Serum.—A glossy culture of Strain New York V was obtained which failed to kill mice in a dose of 0.5 cc. Rabbits were immunized with this culture and a serum was obtained which agglutinated the glossy culture up to a dilution of 1 in 2,560. The glossy culture was grown in various dilutions of this serum (5 per cent, 10 per cent, 50 per cent, 100 per cent) for a number of transfers but in no case was there any evidence of reversion—50 per cent serum was selected as the concentration in which the cocci appeared to multiply most freely and the culture was carried 118 transfers in this medium. At the end of the experiment the form of the colonies and the virulence of the culture remained unchanged.

Immune serum prepared against glossy cocci contains antibodies to the two non-type-specific fractions, P and C. In the following experiment the influence

of pure anti-P serum on glossy cultures of four strains was tested. High titer anti-P serum was prepared by immunizing rabbits with purified nucleoprotein extracted from hemolytic streptococci. The glossy variant forms of four strains (S43, S23, C203, London) were cultivated for twelve transfers in a 10 per cent dilution of this serum. This treatment did not alter the colony forms of the cultures although the virulence of two strains (S23 and London) was definitely increased. The quantities of type-specific substance, however, which could be extracted from the cocci of all four strains remained unchanged.

3. Subcutaneous Inoculation of Mice with Glossy Cultures in Combination with the Homologous Matt Cocci Killed by Heat.—A few experiments have been done with one of our glossy strains (Henson) in an attempt to revert this culture to the matt form by the technique devised by Griffith for the reversion of R pneumococci to the S form. A heavy suspension of the matt culture was prepared by heating, at 60°C. for 30 minutes, the deposit from 50 cc. of culture, concentrated to 2 cc. 0.5 cc. of the heat-killed suspension was mixed with 0.05 cc. of living glossy culture and injected subcutaneously into a mouse. Cultures from the lesion in the mouse contained both matt and glossy colonies although controls indicated that the matt organisms of the heated suspension were dead. Unfortunately, this technique, so successful with pneumococci, is not altogether satisfactory for hemolytic streptococci as it has been found impossible to avoid ulceration when large numbers of heat-killed matt organisms are combined with glossy cultures and even small doses of glossy culture alone frequently cause ulceration. Matt colonies isolated from these open ulcers must be viewed with suspicion since they may arise from contaminating cocci, but it seems probable that further work with this technique may yield convincing evidence of the reversion of the glossy cocci to the matt form.

So far as any conclusions can be drawn from the limited number of observations recorded it seems that the glossy variant is a highly stable form but that reversion may occur under certain conditions.

A Comparison of the Toxigenicity of Matt and Glossy Cultures of the Same Strain.

The method used for comparing the toxigenicity, the virulence for mice and the colony appearance of matt and glossy cultures of the same strain was as follows:

Young broth cultures of the different forms of each strain were sown in 50 cc. of tryptic digest broth. After 16 hours incubation the virulence and colony appearance of a sample taken from each culture were determined; and the flasks were then returned to the incubator. The cultures were filtered after 4 days of incubation and the filtrates were tested by injecting 0.1 cc. of diluted filtrate into

TABLE III.

A Comparison of the Toxigenicity and Virulence of Matt and Glossy Cultures of the Same Strain.

	Identification number of patient	Filtrate from matt virulent culture	Filtrate from matt attenuated culture	Filtrate from glossy culture
1. Bronchopneumonia strains				
Strain S43. Active filtrate diluted 1 in 500	1	15 mm. (10^{-7})	15 mm. (10^{-1})	13 mm. (10^{-1})
" " Boiled " " 1 " 500		11 "	10 "	8 "
" " Active " " 1 " 50	1	27 "	23 "	26 "
" " Boiled " " 1 " 50		18 "	9 "	14 "
" S23. Active " " 1 " 100	1	22 " (10^{-7})	—	20 " (10^{-2})
" " Boiled " " 1 " 100		6 "	—	11 "
2. Scarlet fever strains				
Strain C203. Active filtrate diluted 1 in 1,000	1	28 " (10^{-7})	—	20 " (less than 10^{-1})
" " Boiled " " 1 " 1,000		2 "	—	7 "
" N. Y. V. Active " " 1 " 1,000	2	18 " (10^{-5})	—	19 " (10^{-1})
" " Boiled " " 1 " 1,000		0		0
" " Active " " 1 " 1,000	3	26 mm. (10^{-5})	—	32 mm. (10^{-1})
" " Boiled " " 1 " 1,000		0	—	0
" " Active " " 1 " 1,000	4	10 mm. (10^{-5})	—	10 mm. (10^{-1})
" " Boiled " " 1 " 1,000		0	—	0
" " Active " " 1 " 1,000	5	11 mm. (10^{-5})	—	18 mm. (10^{-1})
" " Boiled " " 1 " 1,000		0	—	0

The figures in brackets give the virulence for mice of the cultures from which the filtrates were prepared.
0 indicates no reaction; — indicates not tested.

the skin of one, or more, known positive reactors. Table III gives the dimensions of the reactions which followed the injection of active filtrate and of the same filtrate after heating in boiling water for 2 hours. Measurements, which are given in mm. representing the average diameter of the skin reactions, were taken 24 hours after injection. The figures in brackets give the virulence for mice of the cultures from which the filtrates were prepared.

The two strains isolated from cases of bronchopneumonia produced weak toxic filtrates in comparison with the scarlet fever strains and were therefore used in greater concentration.

It will be seen from Table III that the filtrates from the different forms of each strain caused approximately equal reactions and that no correlation could be established between virulence and toxigenicity.

DISCUSSION.

It may be stated as a broad generalization that the type-specific substance M is present in the potentially virulent organisms comprising the matt variety of colony and that it is not present in the avirulent variant cocci which form glossy colonies. This is analogous to the invariable presence of the soluble specific substance S in virulent cultures of pneumococci and its absence from avirulent R cultures, but here the analogy breaks down as far as virulence for mice is concerned, since it is possible to prepare matt cultures of hemolytic streptococci which contain large quantities of the type-specific substance M and yet are avirulent for mice.

One of the most striking characteristics of hemolytic streptococci is the difficulty which has always been experienced in securing highly virulent cultures of a large number of strains. This is undoubtedly due in part to the fact that the glossy variant is a highly stable avirulent form but even when we exclude this variant form and confine our attention to the matt or potentially virulent varieties we are still unable to secure highly virulent cultures with any degree of ease. The behavior of matt attenuated cultures undergoing mouse passage is frequently capricious; virulence generally rises to a moderate level after the initial ten, or twenty passages and it may then increase suddenly, or it may gradually increase after many more passages, or it may remain for an indefinite period in a state of mediocrity. This is in contrast to pneumococci which appear to be either rough and avirulent for mice or smooth and of maximal virulence for this

species. The virulence of pneumococci appears to be intimately associated with the presence or absence of the soluble specific substance S; in the case of hemolytic streptococci, however, virulence is not entirely dependent on the presence or absence of the type-specific substance M; some additional unknown factor is operative. Glossy variants, when fully degraded, contain no type-specific substance and are avirulent for mice; matt organisms occur in two forms equally rich in type-specific substance—one of these forms is no more virulent for mice than the glossy variant, the other is highly virulent.

It is possible that this contrast between pneumococci and hemolytic streptococci may be partly due to differences in bacterial structure. In the case of pneumococci the soluble specific substance S is disposed in a capsular layer over the surface of the organism; but microscopic examination of hemolytic streptococci gives no information as to the situation of the type-specific substance M in the bacterial bodies; and it is possible that the distribution of this substance throughout the organisms may render it less accessible and therefore less susceptible to external influences.

Certain strains of hemolytic streptococci exhibit an unexpected stability in all forms. Cultures which are partially degraded so that they contain only small quantities of type-specific substance may be passed through a number of mice without any accumulation of type-specific substance and without any alteration of virulence. Conversely, when the virulence of a matt strain has become established it is difficult to reduce the culture to the matt attenuated state and at the same time to avoid conversion to the glossy variant form.

No relationship could be established between toxigenicity and virulence; in some instances highly virulent matt cultures produced weak toxic filtrates and the glossy variant avirulent forms were equally toxigenic; in other instances relatively avirulent matt strains produced highly toxic filtrates.

It appears therefore that virulence is not determined by toxigenicity and is not entirely dependent on the presence or absence of type-specific substance although cultures which have lost their type specificity are invariably avirulent.

An unknown factor determines whether hemolytic streptococci,

which contain their full quota of type-specific substance, are virulent or attenuated.

SUMMARY.

Hemolytic streptococci, when freshly isolated from pathogenic lesions, form characteristic matt colonies and contain the type-specific substance M.

Two varieties of matt cultures, equally rich in type-specific substance, can be distinguished by the virulence of the organisms for mice: (1) the matt virulent variety, (2) the matt attenuated variety.

The matt forms of hemolytic streptococci can be degraded to a third variety which forms glossy colonies and is always relatively avirulent. This is accomplished by prolonged cultivation on artificial media, by selection of colonies or by cultivation in homologous anti-M serum. In the process of degradation the cocci lose the major part of their type-specific substance but complete disappearance of type-specific substance rarely occurs.

The glossy variant form, when fully degraded, is highly stable; but glossy cultures which have retained some type-specific substance can occasionally be reverted to the original matt form.

Toxic filtrates from matt and glossy cultures are approximately equal in skin reactivity.

No relationship appears to exist between virulence and toxigenicity.

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ANTIGENIC DIFFERENCES BETWEEN MATT HEMOLYTIC STREPTOCOCCI AND THEIR GLOSSY VARIANTS.

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In a previous communication (1) it was shown that hemolytic streptococci in the matt form contain the type-specific substance M and that when matt cultures are degraded to the glossy variant form the organisms lose their type-specific substance. The antigenic differences between matt and glossy cultures, which are described in the present paper, were demonstrated with four different strains of hemolytic streptococci.

Owing to individual differences between the four strains it is essential to describe each series of experiments separately.

A. Strain S43.

This strain was isolated from a case of bronchopneumonia and had been kept in stock cultures for 10 years before our work was commenced. In spite of this long interval, however, the culture was entirely composed of matt colonies. Virulence tests proved that 0.1 cc. killed mice regularly in 24 hours but mice receiving 0.01 cc. survived indefinitely.[1]

The culture was, therefore, in the matt attenuated state and efforts were made to obtain the matt virulent form and the glossy variant so

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[1] A standard technique was used in all the virulence tests recorded in this paper. Cultures were sown in tryptic digest broth and incubated for 16 hours. Tenfold dilutions of culture, in infusion broth, were injected intraperitoneally into a series of white mice, each mouse receiving 0.5 cc. of the appropriate dilution. In some cases counts were made by plating dilutions in blood agar and although this was not done as a routine in every test, a sufficient number of observations were made to show that the dose representing 0.000001 cc. of culture usually contained about 100 organisms.

that the three forms of the same strain could be compared and their antigenic relationships established.

The matt virulent form was secured by passing the original culture through a series of mice (0.1 cc. or 0.2 cc. of peritoneal washings being transferred directly from mouse to mouse), and, although there was an initial increase of virulence, after the first few passages, it required 51 passages before 0.000001 cc. of culture could be relied upon to kill mice regularly.

It was even more difficult to obtain the glossy variant from this highly stable matt strain, as prolonged subcultivation on agar slants did not alter the typical

TABLE I.

Precipitin Reactions of Extracts of S43 Matt Virulent, S43 Matt Attenuated and S43 Glossy with the Homologous Pure Anti-M Serum from Which Non-Type-Specific Antibodies Had Been Removed by Absorption with a Heterologous Strain.

Anti-M serum absorbed with heterologous strain*	Volume of extracts†	Precipitates with extracts from		
		S43 matt virulent	S43 matt attenuated	S43 glossy
0.2 cc.	0.3 cc.	+++	+++	−
0.2 cc.	0.1 cc.	++	++	−
0.2 cc.	0.025 cc.	+	+	−

* Serum R166, against Strain S43 matt attenuated.

† These volumes of crude HCl extract were made up to 0.3 cc. with saline.

The tests were incubated 2 hours at 37°C. and read after standing in the ice box overnight.

+++, ++, + represent degrees of precipitate.

− represents no precipitate.

matt appearance of the colonies. The original culture was, therefore, grown in undiluted, high titer anti-M serum, prepared by immunizing rabbits with the matt attenuated form, and after sixteen transfers in this medium the appearance of the colonies seemed to indicate that the culture had been reduced to the glossy state.[2] Table I shows the precipitin reactions of HCl extracts prepared from the three forms of culture with pure anti-M serum previously absorbed with a heterologous strain to remove antibodies to P and C.

Extracts of the matt virulent and of the matt attenuated cultures formed equally heavy precipitates with anti-M serum but a similar extract of the glossy

[2] Strain S43 in the matt state formed typical matt colonies, but colonies of the variant culture were characterized by a heaped up center surrounded by a wide flat margin and they never assumed the typical glossy appearance.

culture did not cause any precipitation.[3] This experiment showed that the variant contained little, if any, type-specific substance but subsequent examination of a concentrated extract proved that the culture was not entirely free from the type-specific fraction. An HCl extract was made from the deposit of 9 liters of culture, it was then concentrated by precipitation with alcohol and redissolved in 5 cc. of saline. This concentrated and purified extract caused some precipitation with the pure anti-M serum showing that the culture still retained traces of type-specific substance. Further attempts to reduce this strain to a completely degraded state were unsuccessful; colony selection was combined with more than 120 transfers in immune serum but even after this treatment traces of type-specific substance could still be detected.

Three kinds of antiserum were prepared by immunizing three groups of rabbits with the three forms of Strain S43. Four rabbits were included in each group and all received equal doses of the appropriate cultures on the same days—the whole series being finally bled on the same day. The immune sera were used for precipitin reactions and for mouse protection tests; in the latter tests, 0.5 cc. of serum was injected into the peritoneal cavity of a series of mice and the degree of passive immunity conferred was tested, on the following day, by inoculating the mice intraperitoneally with graduated doses of virulent culture.

Precipitin Tests.—Table II gives the results of precipitin reactions with the three kinds of sera and purified solutions of the nucleoprotein P, the carbohydrate C and the homologous type-specific substance M.

The non-type-specific fractions P and C precipitated the three kinds of sera to a similar extent though, as usual, the anti-glossy sera contained, in the aggregate, an excess of C antibody. The type-specific substance M, in contrast to the P and C fractions, formed heavy flocculent precipitates with the anti-matt sera but only traces of precipitate with the anti-glossy sera. The appearance of these traces of precipitate confirmed the observation, already noted above, that the S43 glossy form was not completely degraded by showing that it

[3] Crude HCl extracts of glossy cultures when fully degraded, are free from type-specific substance but they contain the non-type-specific fractions P and C and will consequently precipitate *unabsorbed* antibacterial serum prepared against any strain of hemolytic streptococcus. Precipitates due to the C fraction are easily recognized as they appear late and form compact discs quite unlike the flocculent precipitates of the type-specific fraction which begin to appear as soon as the extract comes in contact with the serum. Precipitates due to the nucleoprotein P are less flocculent than those of the type-specific fraction M and appear as a diffuse cloud; the use of absorbed serum is, however, necessary to distinguish between these two forms of precipitate.

TABLE II.

Precipitin Tests on Three Kinds of Sera, Prepared against the Three Forms of Strain S43 (Matt Virulent, Matt Attenuated and Glossy) with Purified Solutions of Nucleoprotein P, Carbohydrate C and Homologous Type-Specific Substance M.

	Volume of extracts and concentration of P*	Sera prepared with matt virulent culture				Sera prepared with matt attenuated culture			Sera prepared with glossy culture			
		A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11
Purified solution of homologous type-specific substance M from S43	0.4 cc.	++	++	+++	++	++	+++±	+±	±	±	±	+
	0.1 cc.	+±	++	++±	++±	++±	+++	++	±	+	±	+
	0.025 cc.	+±	+	++	+	++	+	+±	±	+	±	+
Purified solution of species-specific carbohydrate C	0.4 cc.	++	++	±	++	+	+±	+	+±	+++	+++	++±
	0.1 cc.	++	++	+	++	++	++±	+±	++	++±	+++	++
	0.025 cc.	+	+±	+	+±	+±	+±	±	+±	+±	+±	+
Purified solution of nucleoprotein P	1 in 1,000	+	+	+	+	+	+	±	+	+	+	+
	1 in 4,000	+	+	+	+	+	+	−	+	+	+	±
	1 in 16,000	−	±	±	±	±	+	−	−	−	±	±

* These volumes were made up to 0.4 cc. with saline, and 0.1 cc. of serum was added to each tube.

produced traces of type-specific antibody when used as an antigen for immunizing rabbits.

TABLE III *a*.

Passive Protection of Mice against S43 Matt Virulent by Three Kinds of Sera, Prepared against S43 Matt Virulent, S43 Matt Attenuated and S43 Glossy.

Test doses of virulent culture	Control normal mice	Sera prepared with matt virulent culture				Sera prepared with matt attenuated culture			Sera prepared with glossy culture			
		A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11
0.00001 cc.	†72	S	S	See	S	S	See	S	S	S	†24	S
0.0001 cc.	†24	S	S	Table	S	S	Table	†77	†90	S	†21	†27
0.001 cc.	†21	S	S	III *b*	S	†21	III *b*	S	S	†22	†21	S
0.01 cc.	—	S	S		†22	†21		S	†21	†21	†21	†21
0.1 cc.	—	†21	†29		†24	†21		S	†21	†21	†21	†21

In all tables the following symbols are used.

S indicates that the mice survived for 8 days and were then discarded.

† indicates death of mice.

Numerals indicate number of hours between time of injection and finding mice dead.

— indicates that the test was not done.

The mice received 0.5 cc. of serum intraperitoneally and 24 hours later test doses of virulent culture were injected into the peritoneal cavity.

TABLE III *b*.

Passive Protection of Mice against S60 Matt Virulent (Same Type as S43) by Three Kinds of Serum Prepared against S43 Matt Virulent, S43 Matt Attenuated and S43 Glossy.

Test doses of virulent culture	Control normal mice	Serum prepared with S43 matt virulent culture A3	Serum prepared with S43 matt attenuated culture A6	Serum prepared with S43 glossy culture A11
0.000001 cc.	†45	S	S	S
0.00001 cc.	S	†45	S	†69
0.0001 cc.	†45	S	S	†45
0.001 cc.	†21	S	S	†23
0.01 cc.	†21	S	S	†21
0.1 cc.	†21	†21	†21	S

Passive Protection of Mice.—Table III *a* shows the degree of passive immunity conferred on mice by these sera against a virulent culture

of the homologous strain and Table III *b* shows the protection against a virulent culture of S60, a strain identified by Dochez, Avery and Lancefield (2) as belonging to the same type as S43.[4]

TABLE IV *a*.

Active Immunization of Mice against S43 Matt Virulent by Vaccines Prepared with S43 Matt Virulent, S43 Matt Attenuated and S43 Glossy.

Test doses of virulent S43 culture	Control normal mice	Mice previously inoculated with S43 vaccines*			Controls Mice previously inoculated with heterologous vaccine prepared from S23 matt virulent and with broth	
		Vaccine prepared with matt virulent culture	Vaccine prepared with matt attenuated culture	Vaccine prepared with glossy culture	Vaccine prepared with heterologous matt virulent culture	Inoculated with broth
0.000001 cc.	†31	S	S	†24	†28	†71
0.00001 cc.	†28	S	†29	†23	†23	†27
0.0001 cc.	†29	S	S	†23	†23	†23
0.001 cc.	†23	†80	†23	†23	†23	†23
0.01 cc.	†23	†23	†23	†23	†23	†23
0.1 cc.	†23	S	†47	†23	—	†23

* Vaccines = undiluted 16 hour broth cultures heated at 56°C. for 1 hour.

TABLE IV *b*.

Showing that the Actively Immune Mice Surviving the Test Recorded in Table IV a Were Not Protected against a Matt Virulent Culture of a Heterologous Strain S23.

Test doses of virulent S23 culture	Control normal mice	Mice protected with S43 matt vaccines which had survived test doses of S43 matt virulent
0.000001 cc.	S	†23
0.00001 cc.	†23	†23
0.0001 cc.	†144	†23
0.001 cc.	†23	†23
0.01 cc.	†23	†23
0.1 cc.	†8	†23

The sera prepared against the matt virulent and matt attenuated cultures afforded an equal amount of protection to mice and the

[4] Most of the protection tests recorded in this paper were only done once and no attempt has been made to correct obvious discrepancies due to the natural resistance of individual mice by repeating the tests.

anti-glossy sera also showed some protective power. The small degree of protection conferred on mice by the anti-glossy sera corresponds to the small quantity of type-specific antibody contained in the sera (see Table II) and this coincidence becomes significant when we observe, from the study of strain S23 that serum, prepared with completely degraded glossy cultures which are devoid of type-specific substance, does not contain any type-specific antibody and does not protect mice.

Active Immunization of Mice.—Table IV records the results obtained when mice, actively immunized with the matt virulent, matt attenuated and glossy cultures, were tested for immunity.

Tryptic digest broth cultures of the three forms of Strain S43 were incubated for 16 hours and then killed by heating at 60°C. for an hour. These heat-killed cultures, undiluted and suspended in the broth in which they had grown, were inoculated intraperitoneally into three series of mice. During the 1st week the mice received 0.1 cc. doses on 4 successive days, during the 2nd week four 0.2 cc. doses and during the 3rd week four 0.4 cc. doses. 10 days after the final inoculation the active immunity of the mice was tested by intraperitoneal injection of graduated doses of the matt virulent culture. The controls for this experiment were (1) a series of normal untreated mice, (2) a series of mice inoculated with corresponding doses of tryptic digest broth, (3) a series of mice treated with the matt virulent vaccine of a heterologous strain, S23.

The mice vaccinated with the matt cultures in both the virulent and attenuated forms showed evidence of protection; but mice vaccinated with the glossy form were not protected against infection with the matt virulent cocci.

The specificity of active immunization is also demonstrated by this experiment. Vaccination with a heterologous matt virulent Strain S23 did not afford any protection against Strain S43 and Table IV *b* shows that there was no cross-immunity when protected mice surviving from the first experiment and immune to S43 were tested 10 days later for immunity to S23.

B. Strain S23.

This strain, isolated from a case of bronchopneumonia at the same time as Strain S43, had been used by Andrewes, Derick and Swift (3) as the test organism for protection experiments, and in the course

of their work its virulence had been increased by passage through a number of mice.

Virulence tests showed that 0.000001 cc. of culture killed mice regularly and occasionally 0.0000001 cc. and even 0.00000001 cc. proved a fatal dose.

The colonies had an undulating contour which made observations on the light-reflecting properties of their surfaces difficult and this peculiarity persisted when the strain was converted to the glossy state. Cultures of the virulent form usually contained many pseudo-glossy colonies and, although a large number of plate cultures were examined, it was only rarely that either matt or glossy colonies appeared in typical forms.

Owing to these peculiarities we relied entirely on precipitin tests, by which the quantity of type-specific substance in a culture can be assessed, to judge when this strain was completely degraded to the variant form. The matt virulent strain was cultivated in homologous high titer anti-M serum and after 55 transfers in 50 per cent serum, concentrated extracts of the culture did not precipitate the homologous anti-M serum; and a single mouse passage did not cause the reappearance of type-specific substance. This variant culture, which, measured by the absence of type-specific substance, was completely degraded, was nevertheless partially virulent, 0.01 cc. being the M.L.D. for mice.

It was difficult to obtain a matt attenuated culture as the peculiarities of the strain made selection of colonies impossible. After nine transfers in homologous immune serum, containing only a small amount of type-specific antibody, the virulence of the culture was reduced from 0.000001 cc. to 0.001 cc. although the quantity of type-specific substance, measured by precipitin reactions, remained unchanged. This culture probably represented the matt attenuated form of Strain S23 as it was only slightly more virulent than the glossy variant; but owing to its high virulence, compared with the matt attenuated cultures of other strains, it was not used in our experiments.

Precipitin Tests.—Two series of rabbits were immunized with the matt virulent and glossy forms of Strain S23; Table V shows the precipitin reactions of their sera. It will be seen that all the sera

TABLE V.

Precipitin Tests on Two Kinds of Sera, Prepared against S23 Matt Virulent and S23 Glossy, with Purified Solutions of Nucleoprotein P, Carbohydrate C and Homologous Type-Specific Substance M.

	Volume of extracts and concentration of P	Sera prepared with matt virulent culture					Sera prepared with glossy culture				
		B1	B3	B4	B5	B6	B7	B8	B9	B10	B12
Purified solution of homologous type-specific substance M from S23	0.4 cc.	++	++	++	±	+++	−	−	−	−	−
	0.1 cc.	++±	++±	++±	±	+++	−	−	−	−	−
	0.025 cc.	+±	++	+	+	+	−	−	−	−	−
Purified solution of species-specific carbohydrate C	0.4 cc.	−	+±	−	−	−	+	±	−	±	+
	0.1 cc.	−	++	−	−	−	++	++	++	+	+±
	0.025 cc.	−	++±	−	−	−	++±	++	++	+	++
Purified solution of nucleoprotein P	1 in 1,000	±	±	±	−	±	−	±	++	±	±
	1 in 4,000	−	−	−	−	±	−	−	+±	−	−
	1 in 16,000	−	−	−	−	−	−	−	±	−	−

prepared against the matt virulent culture were precipitated by an extract containing the homologous type-specific substance but the sera prepared against the glossy variant remained perfectly clear

TABLE VI.

Absorption of Anti-Matt and Anti-Glossy Sera with the Homologous Matt and Glossy Cultures. Strain S23.

	Volumes of extracts	Serum B6 prepared with matt virulent culture of Strain S23			Serum B9 prepared with glossy culture of Strain S23		
		Control unabsorbed	Absorbed with S23 matt virulent	Absorbed with S23 glossy	Control unabsorbed	Absorbed with S23 matt virulent	Absorbed with S23 glossy
Purified solution of homologous type-specific substance M from S23	0.4 cc.	++±	−	++±	−	−	−
	0.1 cc.	++±	−	++±	−	−	−
	0.025 cc.	+	−	+	−	−	−
Purified solution of species-specific carbohydrate C	0.4 cc.	−	−	−	−	−	−
	0.1 cc.	−	−	−	+±	−	−
	0.025 cc.	−	−	−	++	−	−

TABLE VII.

Passive Protection of Mice against S23 Matt Virulent by Two Kinds of Serum Prepared against S23 Matt Virulent and S23 Glossy.

Test doses of virulent culture	Control normal mice	Sera prepared with matt virulent culture					Sera prepared with glossy culture				
		B1	B3	B4	B5	B6	B7	B8	B9	B10	B12
0.0000001 cc.	†67	−	−	S	−	S	†30	†21	†31	†23	†29
0.000001 cc.	†23	S	S	S	S	S	†21	†24	†25	†20	†29
0.00001 cc.	†23	†23	†23	S	†23	S	†21	†21	†26	†20	†23
0.0001 cc.	†23	S	S	S	†26	S	†21	†21	†23	†20	†23
0.001 cc.	−	†28	S	†96	S	S	†21	†21	†23	†20	†23
0.01 cc.	−	†21	†21	†27	†21	†72	†21	†21	†23	†20	†23
0.1 cc.	−	†21	†21	†21	†21	S	†21	†21	−	†20	†23

when mixed with the same extract. Both kinds of immune sera were precipitated by the non-type-specific fractions P and C, the anti-glossy sera being as usual more uniformly rich in antibody to the carbohydrate fraction C.

The anti-matt serum (B6) and the anti-glossy serum (B9) were chosen from the two groups of sera for absorption experiments. Both sera were absorbed with homologous matt virulent cocci and also with homologous glossy cocci. Table VI shows the result of this experiment.

The type-specific antibody was completely removed from the anti-matt serum by absorption with the homologous matt culture but it was unaffected by absorption with the homologous glossy culture.

The anti-glossy serum contained no type-specific antibody but its non-type-specific antibody was completely removed by absorption with both forms of the homologous strain.

TABLE VIII.

Active Immunization of Mice against S23 Matt Virulent by Vaccines Prepared with S23 Matt Virulent and S23 Glossy.

Test dose of virulent S23 culture	Control normal mice	Mice previously inoculated with S23 vaccines		Controls Mice previously inoculated with heterologous matt virulent Vaccine S43 and with broth	
		Vaccine prepared with matt virulent culture	Vaccine prepared with glossy culture	Vaccine prepared with heterologous matt virulent culture	Inoculated with broth
0.000001 cc.	S	S	†24	†41	†41
0.00001 cc.	†63	†41	†21	†41	†26
0.0001 cc.	†23	†41	†22	†22	†21
0.001 cc.	†21	†110	†21	†21	†41
0.01 cc.	†21	†17	†17	†17	†17
0.1 cc.	†17	†13	†17	†22	†17

Passive Protection of Mice.—The sera prepared against the two forms of Strain S23 were used for mouse protection experiments with the homologous matt virulent culture as the test organism.

All the sera prepared against the matt virulent form afforded some degree of protection to mice but the anti-glossy sera did not confer any protection.

Active Immunization of Mice.—Two series of mice were inoculated with vaccines prepared from the matt and glossy forms of Strain S23; the dosage and technique were the same as those used in a similar experiment, previously recorded, with Strain S43. Controls con-

sisted of a series of normal mice, a series of mice inoculated with a heterologous matt virulent vaccine and a series of mice inoculated with broth (Table VIII).

In this experiment vaccines prepared from both forms of Strain S23 failed to confer immunity on the mice.

C. Strain C203.

This strain, from the collection of the Laboratories of the New York State Department of Health, was originally isolated from a

TABLE IX.

Precipitin Tests on Two Kinds of Sera, Prepared against C203 Matt Virulent and C203 Glossy with Purified Solutions of Nucleoprotein P, Carbohydrate C and Homologous Type-Specific Substance M.

	Volume of extracts and concentration of P	Sera prepared with matt virulent culture			Sera prepared with glossy culture			
		C1	C2	C3	C4	C5	C6	C7
Purified solution of homologous type-specific substance M from C203	0.4 cc.	+++±	++	++	±	±	−	±
	0.1 cc.	++	+±	+	−	−	±	−
	0.025 cc.	+	+	±	−	−	−	−
Purified solution of species-specific carbohydrate C	0.4 cc.	−	−	−	−	+	−	+±
	0.1 cc.	−	−	−	++	+++±	−	+++
	0.025 cc.	±	−	−	++±	++	−	++±
Purified solution of nucleoprotein P	1 in 1,000	+±	±	+	++	++±	+	++±
	1 in 4,000	+	±	±	+±	+	±	+±
	1 in 16,000	−	−	−	±	±	−	±

case of scarlet fever and had been kept in stock culture for a considerable time before our experiments commenced. It was found to be entirely composed of typical matt colonies; and virulence tests showed that the M.L.D. for mice was 0.0000001 cc. or 0.00000001 cc.

The glossy variant form of this strain was obtained by selection of colonies combined with cultivation in 50 per cent homologous anti-M serum of high titer. After 27 transfers the virulence for mice had fallen from 0.0000001 cc. to 0.1 cc. and the culture formed typical glossy colonies; but traces of type-specific substance could still be

detected in unconcentrated HCl extracts. Continued efforts to secure a completely degraded culture did not cause any further decrease in the quantity of type-specific substance which the organisms contained.

Attempts to reduce this strain to the matt attenuated form were unsuccessful as any reduction in virulence was always accompanied by a partial loss of type-specific substance and by the appearance of atypical matt colonies which tended to assume the characteristics of glossy colonies.

Precipitin Tests.—Rabbits were immunized with the matt virulent and glossy forms of this strain; Table IX gives the precipitin reactions of the immune sera.

In this instance a departure was made from the technique used for immunizing rabbits with the other three strains; the organisms were washed to remove the toxin as the use of unwashed broth cultures of highly toxic strains involved the loss of a relatively large proportion of rabbits during immunization.

The results of precipitin tests were similar to those previously recorded in reference to other strains; the sera prepared with the matt virulent organisms gave good precipitates with the homologous type-specific substance M but only traces of precipitate with the non-type-specific fractions C and P; on the other hand sera prepared with glossy organisms gave only traces of precipitate with the type-specific substance M and comparatively good precipitates with the non-type-specific substances C and P.

The traces of type-specific antibody in the anti-glossy sera were a natural sequel of failure to secure this strain in the completely degraded form.

Passive Protection of Mice.—Table X*a* shows the results of protection tests with the different kinds of sera. All the anti-matt sera showed evidence of protective power and the anti-glossy sera with one exception also protected mice to some extent. This protection by sera prepared against the glossy form of Strain C203 is in harmony with the results of precipitin tests and with the original observation that the culture was not fully degraded but still contained type-specific substance. Table X*b* shows the specificity of protection by the anti-glossy sera. On reference to Table X*a* it will be seen that

TABLE X *a*.

Passive Protection of Mice against C203 Matt Virulent by Two Kinds of Sera Prepared against C203 Matt Virulent and C203 Glossy.

Test doses of virulent culture Strain C203	Control normal mice	Sera prepared with matt virulent culture			Sera prepared with glossy culture				Control mice inoculated with normal rabbit sera	
		C1	C2	C3	C4	C5	C6	C7	N1	N2
0.0000001 cc.	†24	S	S	S	†26	†25	S	S	S	†22
0.000001 cc.	†21	†24	S	†120	†22	S	S	S	†24	†28
0.00001 cc.	†21	S	S	S	†21	†25	S	†21	†21	†26
0.0001 cc.	†21	S	†168	S	†21	†25	†69	†21	†21	†21
0.001 cc.	†21	†24	†21	S	†21	S	†93	†21	†21	†21
0.01 cc.	—	†21	†21	†24	†21	†21	†21	†21	†21	†21

TABLE X *b*.

Specificity of Passive Protection of Mice by Anti-Glossy (C203) Serum.

Test doses of virulent culture Strain S23	Control normal mice	Serum prepared with glossy culture of Strain C203 C6
0.0000001 cc.	†53	†51
0.000001 cc.	†51	†22
0.00001 cc.	†22	†26
0.0001 cc.	†22	†56
0.001 cc.	†22	†22
0.01 cc.	—	†22

TABLE XI.

Active Immunization of Mice against C203 Matt Virulent by Vaccines Prepared with C203 Matt Virulent and C203 Glossy.

Test dose of virulent C203 culture	Control normal mice	Mice previously inoculated with C203 vaccines		Mice previously inoculated with heterologous matt virulent Vaccine S43 and with broth	
		Vaccine prepared from matt virulent culture	Vaccine prepared from glossy culture	Vaccine prepared from heterologous matt virulent culture	Inoculated with broth
0.0000001 cc.	†25	S	—	†40	†40
0.000001 cc.	†21	S	†24	†20	†25
0.00001 cc.	†22	S	†64	†16	†18
0.0001 cc.	†16	S	†20	†21	†16
0.001 cc.	†16	S	†16	†16	†16
0.01 cc.	†16	S	†17	†16	†16
0.1 cc.	†16	†20	†16	†16	†16

Serum C6 was probably the best anti-glossy serum for protection against the homologous matt virulent form of Strain C203; a series of mice was, therefore, injected with this serum and their immunity to a heterologous matt virulent Strain S23 was subsequently tested. Table X*b* shows that Serum C6 afforded no protection against the heterologous Strain S23.

Active Immunization of Mice.—Mice were vaccinated with the different forms of Strain C203 in the manner previously described; and their immunity was tested by inoculating graduated doses of a matt virulent culture of the same strain.

Table XI shows that vaccination with matt virulent organisms produced immunity against subsequent infection by the same strain; but vaccination with homologous glossy organisms, or with heterologous matt virulent organisms did not cause any immunity.

D. Strain London.

This strain, isolated by blood culture from a case of puerperal septicemia, had been kept in stock for about a year before our experiments began; and plate cultures showed that it was composed of a mixture of matt and glossy colonies. A pure matt culture, capable of killing mice in a dose of 0.01 cc., was obtained by selecting a suitable colony and the matt virulent form (M.L.D. 0.000001 cc.) was then prepared by passing this culture through 47 mice.

The matt attenuated culture, which was entirely composed of typical matt colonies indistinguishable from those of the virulent form and failed to kill mice when 0.5 cc. was injected intraperitoneally, was obtained by combining the selection of typical matt colonies with cultivation at 41°C.

The glossy variant was obtained by daily subcultivation of the original strain on agar slants without any selection of colonies or cultivation in immune serum. After 148 transfers on agar the culture was entirely composed of typical glossy colonies, and it was avirulent for mice (M.L.D. 1.0 cc.); but precipitin tests with concentrated HCl extracts of the organisms showed that this culture still retained traces of the type-specific substance M.

Precipitin Tests.—Table XII gives the precipitin reactions of the sera of three sets of rabbits immunized with the three forms of this

TABLE XII.

Precipitin Tests on Three Kinds of Sera, Prepared against the Three Forms of Strain London (Matt Virulent, Matt Attenuated and Glossy) with Purified Solutions of Nucleoprotein P, Carbohydrate C and Homologous Type-Specific Substance M.

	Volume of extracts and concentration of P	Sera prepared with matt virulent culture					Sera prepared with matt attenuated culture						Sera prepared with glossy culture					
		D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	D16	D17
Purified solution of homologous type-specific substance M	0.4 cc.	++	+±	++	++	++	+±	+	+±	±	+	+	±	−	±	±	−	±
	0.1 cc.	+±	+	+±	+±	++	+	±	+±	±	+	+	−	−	−	−	−	−
	0.025 cc.	+	±	−	±	±	−	−	−	−	−	±	−	−	−	−	−	−
Purified solution of species-specific carbohydrate C	0.4 cc.	±	−	−	−	−	−	++	−	−	−	−	−	+±	−	++	++	−
	0.1 cc.	++	−	−	−	+	−	++	−	−	+±	−	±	++±	++	+++	+++	++±
	0.025 cc.	++±	−	−	+±	++	−	++	−	−	++	++	++	++±	++±	+++	++±	++±
Purified solution of nucleoprotein P	1 in 1,000	+±	+	+±	+	+	+	+	±	±	+	+	+	+	+	+	+	+
	1 in 4,000	+±	+	±	+	++	+	+	±	−	−	+	+	±	±	±	±	±
	1 in 16,000	+	±	−	±	+±	−	±	−	−	−	−	−	−	±	−	−	−

TABLE XIII.

Passive Protection of Mice against Matt Virulent Form of Strain London by Three Kinds of Sera Prepared against the Three Forms of Strain London (Matt Virulent, Matt Attenuated and Glossy).

Test dose of virulent culture	Control normal mice	Sera prepared with matt virulent culture					Sera prepared with matt attenuated culture						Sera prepared with glossy culture						Control mice inoculated with normal rabbit serum
		D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	D16	D17	
0.0000001 cc.	S	S	S	S	S	S	S	S	S	S	S	S	†69	†69	†45	†45	S	S	†30
0.000001 cc.	S	S	†45	†90	S	S	S	S	S	S	S	S	†90	†45	†30	†21	†29	S	†76
0.00001 cc.	†21	S	S	†52	S	S	S	†90	†90	S	S	S	†55	†51	†31	S	†31	†55	†45
0.0001 cc.	†21	S	S	†31	†45	S	S	†45	S	S	S	†90	†99	†45	†21	†31	†29	†30	†53
0.001 cc.	†21	S	†120	S	S	†140	S	†26	S	S	†160	S	†27	†21	†29	†21	†21	†21	†21
0.01 cc.	†21	S	†140	S	S	†26	†26	†26	S	S	†21	†21	†21	†21	†21	†21	†21	†21	†21

strain; and it will be seen that, as in previous similar experiments, the anti-matt sera were relatively rich in antibody to the type-specific substance M; while the anti-glossy sera had a preponderance of antibody to the non-type-specific fraction C.

Passive Protection of Mice.—Table XIII gives the results of protection tests with the sera prepared against the different forms of Strain London: both kinds of anti-matt sera protected mice against infection with the homologous matt virulent organisms but the anti-glossy sera, although they contained traces of type-specific antibody had little protective power.

TABLE XIV.

Active Immunization of Mice against the Matt Virulent Form of Strain London by Vaccines Prepared from the Three Forms of Strain London (Matt Virulent, Matt Attenuated and Glossy).

Test dose of matt virulent culture of Strain London	Control normal mice	Mice previously inoculated with vaccines prepared from Strain London in the following forms			Mice previously inoculated with heterologous vaccine prepared from S23 matt virulent and with broth	
		Matt virulent	Matt attenuated	Glossy	Heterologous matt virulent	Broth
0.0000001 cc.	†41	—	†89	—	†41	†41
0.000001 cc.	†27	†89	†41	†41	†25	†41
0.00001 cc.	†22	†70	†22	†24	†41	†27
0.0001 cc.	†22	S	†22	†22	†24	†17
0.001 cc.	†24	†65	S	†22	†22	†17
0.01 cc.	†17	†22	†17	†22	†17	†17
0.1 cc.	†17	†17	†17	—	†17	—

Active Immunization of Mice.—Table XIV shows the results of vaccinating mice with the three forms of Strain London and with matt virulent organisms of a heterologous strain. In this instance a final dose of 0.2 cc. of heat-killed culture was substituted for 0.4 cc. as used in other experiments because the mice began to lose weight during immunization. In this experiment, although there was little evidence of immunization by any of the vaccines, the mice vaccinated with the matt virulent organisms survived longer than any of the controls.

DISCUSSION.

Matt and glossy cultures of four strains of hemolytic streptococci, belonging to different serological types, were used to immunize rabbits. Only one of the four glossy cultures, Strain S23, was completely degraded to the point at which no trace of type-specific substance could be detected in highly concentrated HCl extracts of the organisms. The glossy cultures of the other three strains were not completely degraded as extracts from these organisms contained traces of the type-specific substance M.

Precipitin tests with the antisera of the four strains gave the following results: (1) All the anti-matt sera, whether prepared with virulent or attenuated organisms, contained antibody to the type-specific substance M. (2) The anti-glossy sera, prepared with cultures which were not fully degraded, were relatively deficient in type-specific antibody and gave either negative or weakly positive precipitin reactions with the type-specific substance M. (3) The five sera prepared with the completely degraded glossy form of Strain S23 did not contain any type-specific antibody. (4) The type-specific antibody was completely removed by absorption with homologous matt organisms but was unaffected by absorption with homologous glossy organisms.

Experiments on passive immunization of mice showed that all the anti-matt sera had some protective power against infection with homologous matt virulent organisms. Some of the anti-glossy sera, prepared against strains which were not fully degraded, also protected mice but none of the anti-glossy sera prepared against the completely degraded glossy form of Strain S23 afforded any protection against infection with the homologous virulent organisms.

No exact parallel was established between the anti-M titer and the protective power of immune sera but high titer anti-M sera usually gave good protection.

It might be supposed that protection against infection with matt organisms by anti-matt sera and the absence of protection by anti-glossy sera merely represented specificity which could be demonstrated between different forms of the same strain and that anti-glossy sera would protect against infection with glossy organisms

better than anti-matt sera. Three of the strains used in this investigation were unsuitable for experiments to test this possibility because the glossy organisms were completely avirulent for mice. The completely degraded glossy variant of Strain S23 was, however, relatively virulent for mice (M.L.D. 0.01 cc.) and comparison was, therefore, made between the protective power of anti-matt and of anti-glossy sera against infection with the homologous glossy organisms. No evidence was obtained that either the anti-matt sera or the anti-glossy sera had any protective power against infection with glossy organisms.

There is considerable evidence that both passive protection and active immunity were antibacterial and not antitoxic; this evidence may be summarized as follows:

1. All the strains produced toxin to some extent but the protective action of the antisera was type-specific; sera prepared with toxigenic strains did not protect against infection with heterologous strains of equal toxigenicity. The immunity due to vaccination was also type-specific.

2. The toxigenicity of the matt form and of the glossy form of Strain S23 was determined quantitatively by intracutaneous tests in human subjects, and it was found that the matt and the glossy forms were approximately equal in their power to produce skin-reactive toxin. In spite of this equality, anti-glossy sera possessed no protective power against infection with homologous matt virulent organisms.

3. The anti-matt sera against Strain C203 were prepared with washed bacteria, and the production of antitoxin was, therefore, limited, but in spite of this limitation the sera protected against the highly toxigenic homologous strain but not against the weakly toxigenic heterologous Strain S23.

4. Active immunization of mice with whole broth cultures of the matt and of the glossy forms of the scarlet fever Strain C203 showed that, although the two forms were approximately equal in toxigenicity, the matt vaccine produced a high degree of immunity while the glossy vaccine produced no immunity against infection with homologous virulent organisms.

These observations, which show that the matt varieties of hemolytic

streptococci are type-specific while the glossy variant forms are not type-specific, are in agreement with the work of Andrewes. He also isolated two varieties of hemolytic streptococci corresponding to our matt and glossy forms, which, in a preliminary communication (4), he designates "rough" and "smooth" with the reservation "that they must not be supposed to correspond with the rough and smooth forms of *B. coli* and Salmonella." Using a special technique, he was able to establish by agglutination and absorption experiments that the "rough" forms exhibit considerable specificity; that the "smooth" forms of different strains are all alike serologically, and that the "rough" and "smooth" forms of a given strain are serologically distinct.

A comparison of his results with those recorded in this paper shows that his type-specific agglutination with hemolytic streptococci of the matt variety is in agreement with our observation that large quantities of the type-specific substance M are found in matt organisms; also his observation that anti-glossy serum agglutinates all strains of hemolytic streptococci, when in the glossy form, with complete impartiality is in agreement with our observation that anti-glossy sera contain more antibody to the non-type-specific fractions than anti-matt sera.

SUMMARY.

The matt and the glossy forms of four strains of hemolytic streptococci were used to immunize rabbits.

Precipitin tests showed that rabbit sera prepared against matt organisms, whether virulent or avirulent for mice, contained type-specific antibody while sera prepared against completely degraded glossy organisms contained no type-specific antibody.

Type-specific antibody was removed from the sera by absorption with homologous matt organisms but was unaffected by absorption with homologous glossy organisms.

Passive protection experiments on mice showed that anti-matt sera were protective and anti-glossy sera non-protective against infection with homologous virulent organisms.

Vaccination of mice with matt organisms rendered them immune to subsequent infection with homologous virulent cultures; but vaccination with glossy organisms established no active immunity.

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ACTIVE AND PASSIVE IMMUNITY TO PNEUMOCOCCUS INFECTION INDUCED IN RABBITS BY IMMUNIZATION WITH R PNEUMOCOCCI.

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In a previous publication (1) the fact was established that rabbits, immunized with degraded, avirulent, non-type-specific pneumococci —so called R strains derived from any one of the three types—acquire a considerable degree of resistance against subsequent infection with virulent Type III pneumococci. Active resistance was demonstrable under these conditions in spite of the fact that the sera of the immunized rabbits contained no type-specific antibodies capable of agglutinating Type III S cells, or of precipitating the soluble specific substance derived from Type III cultures, or of conferring passive protection on mice against Type III infection. It was suggested (1) that this form of active immunity, effective in the absence of demonstrable type-specific antibodies and unrelated to the variety of pneumococcus used for immunization, differed in principle from type-specific immunity. The previous experiments were restricted to the study of the active resistance to Pneumococcus Type III. However, it seemed possible that this form of immunity—induced by immunization with R cells—might be effective against infection with other virulent types of pneumococcus. Consequently, additional studies have been carried on to determine whether the apparently non-type-specific resistance thus induced is as effective against Type I and Type II as against Type III. The results are recorded in this communication. In view of the differences which appear to exist between the form of active immunity stimulated in rabbits by type-specific S organisms and intimately associated with type-specific antibodies, and active resistance induced by prolonged immunization with R cells, further investigation into the nature and mechanism of the latter type of immunity is in progress.

In addition, the work has been extended to include passive immunity in order to determine whether the blood of rabbits immunized with R pneumococci, is capable of conferring protection upon normal animals of the same or of different species. The results of experiments on passive immunity are also included in this report.

Methods.

Antigens.—An R strain originally derived from Type II S culture (designated R_2), an R strain similarly derived from Type I S culture (designated R_1), and a Type III S strain were employed for the immunization of all rabbits used in the experiments on active immunity. Only the R_2 strain was used for immunization in the tests for passive immunity.

Vaccines were prepared from 12 to 14 hour plain broth cultures. The organisms, removed from the broth by centrifugation, were resuspended in physiological salt solution in such proportion that 0.5 cc. of the suspension was equivalent in bacterial content to 1 cc. of original culture, and heat-killed at 56° for 30 minutes.

Technique of Immunization.—The method of immunization, described by Cole and Moore (2), consisted of a total of 18 intravenous injections; 0.5 cc. doses of vaccine were given daily for 6 days followed by a week of rest until the procedure was repeated 3 times. As a rule, the animals were tested 9 to 14 days after the last dose.

Protection Tests.—12 to 14 hour blood broth cultures of virulent pneumococci of Types I, II, and III were used in all tests for active and passive immunity. Type I cultures possessed a maximum virulence for rabbits of 0.000001 cc.; Type II were usually fatal in 0.000001 cc. and always in 0.00001 cc. amounts; Type III killed regularly at 0.0001 cc. In most of the experiments, in order to follow the degree of the bacteremia, blood cultures were taken at frequent intervals during the course of infection as previously described (3).

Rabbits tested for increased resistance to Type I infection had previously been treated as follows: 1 had been immunized with R pneumococci derived from Type I S strain, 15 with R pneumococci derived from Type II S strain, and 3 with Type III S pneumococci. Their sera were tested for the presence of agglutinins for R organisms and for the S forms of the three specific types. In no instance were type-specific agglutinins demonstrable; there were, however, agglutinins for R cells present in titres of 1:640 to 1:1280. Antibody response was similar regardless of organisms used for immunization. That rabbit avirulent strains of Type III stimulate in rabbits antibody formation of the same character as that elicited by R cells has been previously described (4). The infecting dose of Type I pneumococcus, in every instance, represented 10,000 to 100,000 lethal doses. By keeping the test strain at a maximum virulence for rabbits of 0.000001 cc., 0.01 cc., or 0.1 cc. of such culture constituted the dosage of organisms employed.

In each experiment, in addition to the immunized rabbits, normal animals receiving the minimal lethal dose and others the test dose served as controls of the virulence of the culture.

TABLE I.

Active Immunity against Infection with Pneumococcus Type I in Rabbits Immunized with R Strains. (Three Rabbits Immunized with S_{III} Are Also Included.)

Number of rabbits	Immunized with	Infected with Pneumococcus Type III	Route of infection	Number died	Number survived
		cc.			
1	R_I	0.01	Intravenous	0	1
3	S_{III}	0.1	"	1*	0
		0.01	"	0	2
8	R_2	0.1	"	1†	5
		0.01	"	0	2
4	R_2	0.1	Intraperitoneal	1‡	0
		0.01	"	0	3
6	R_2	0.1	Intradermal	0	2
		0.01	"	0	4
Total 22				3	19
9	Normal controls	0.000001	Intravenous	6	0
		0.000001	Intraperitoneal	1	0
		0.000001	Intradermal	2	0
Total				9	0

* Animal died 8 days after infection.

† Animal died 5 days after infection.

‡ Animal died 7 days after infection. Controls receiving test dose of culture died within 36 hours.

Active Immunity.

Rabbits were tested for resistance to infection by the injection of organisms intravenously, intraperitoneally, and intradermally (Table I). Blood cultures were taken at frequent intervals in order to observe the duration and degree of the bacteremia.

Rabbits Infected Intravenously.—Of 12 rabbits infected intravenously, 5 of which received 0.01 cc., and 7 0.1 cc. each of virulent Type I culture, 10 survived. The 2 which finally succumbed, lived 5 and 8 days respectively, whereas normal rabbits receiving the same dose died in 24 to 48 hours. As evidence of the duration of the immunity, 2 of the rabbits in this group were tested 4½ months after the last immunizing dose and found to be resistant. As demonstrated by blood cultures, organisms persisted in the circulation for 3 to 6 days, increasing and decreasing in number irregularly until their final disappearance. Even in the two fatal instances, the animals possessed some degree of partial immunity as revealed by the fact that both lived several days longer than the controls, that neither suffered an overwhelming septicemia, and that at autopsy each showed evidence of attempted localization in the form of pleurisy and pericarditis. A bacteremia characterized by an irregular course was previously shown to occur when R immunized rabbits were infected with a rabbit virulent strain of Type III pneumococcus (1). This form of bacteremia appears to be characteristic of the benign blood infection occurring when rabbits, immunized with R cells, are infected intravenously with any of the specific types of pneumococcus and suggests a similarity in the mechanism of recovery in each instance.

Rabbits Infected Intraperitoneally.—Four rabbits, immunized with R_2 organisms, were infected intraperitoneally; 1 received 0.1 cc. and 3 received 0.01 cc. of Type I pneumococci. The latter 3 animals survived; the one injected with 0.1 cc. lived 7 days. In the 3 animals which survived, only a few bacteria were transiently present in the blood stream. The duration of cocci in the peritoneal cavity was not determined.

Rabbits Infected Intradermally.—Type I organisms in doses of 0.01 cc. and 0.1 cc. were introduced intradermally into 6 rabbits which had previously been immunized with R_2 cells. All 6 animals survived. The local lesion developed rapidly; in 24 to 48 hours it appeared fulminating, usually reddish purple, edematous, spreading ventrally in a well defined and elevated band and forming a boggy pouch of edema over the more dependent portions of the abdominal wall. Areas of ecchymosis were commonly present. Normal rabbits reacted with a similar lesion, although succumbing to the infection. Furthermore, blood cultures revealed a striking difference in the course of the infection in normal and immunized animals. The blood stream of immune rabbits either remained sterile or contained only a few organisms transiently present. In sharp contrast, normal rabbits developed, within a few hours, a blood infection which increased rapidly in severity until death ensued from an overwhelming septicemia. Recently Goodner (5) has reported results obtained following intradermal injection of Type I pneumococci. The lesion which he describes as occurring in normal rabbits is identical with the inflammatory reaction encountered in the animals used in these experiments. Results which have been obtained following the intradermal inoculation of pneumococci into normal, type-specifically immune, and R immunized rabbits will be reported later in a separate communication.

In addition to the rabbits immunized with R organisms and tested for resistance to infection with Type I, 3 rabbits which had received similar preliminary injections were infected intravenously with virulent Type II pneumococci. They survived 10,000 lethal doses. The character of the blood infection and the process of recovery were similar in all respects to those already observed in the case of infection with Types I and III.

From the results of these experiments with Type I and Type II pneumococci, and from those reported (1) using Type III, it may be concluded that adequate immunization of rabbits with R pneumococci stimulates the development of active immunity which is effective against any of the fixed types. A consideration of these results and their possible significance will be presented in the discussion.

Passive Immunity.

In the course of an analysis of the immunity induced by repeated injections of R pneumococci, experiments have been carried out to determine whether this form of resistance is passively transferable. Whole citrated blood and serum of rabbits which have acquired resistance through immunization with R cells have been passively transferred to normal rabbits and also to mice. The R strain used for immunization was derived from Type II S culture. The infecting organisms were virulent S strains of Type I or Type III. These precautions were taken in order to minimize the possible participation of type-specific antibodies. The blood for transfusion was drawn 9 to 14 days after the last immunizing dose.

In the first experiments the procedure was to transfer whole blood or its constituents from resistant to normal rabbits and 24 hours later to inject the recipients with virulent pneumococci. An example of the results obtained following the passive transference of whole citrated blood, plasma, cells, and serum is given in Table II.

A description of this experiment will serve as an illustration of the method employed and the results obtained.

From the ear vein of an R immunized rabbit 20 cc. of blood was allowed to drop into a tube containing 0.5 cc. of a saturated solution of sodium citrate. This made in final dilution approximately a 2 per cent sodium citrate solution. Immediately upon obtaining the desired amount, the blood was injected by means of a syringe into the ear vein of a normal rabbit. In making transfusions from rabbit to

rabbit no precaution was taken with regard to blood grouping. Sometimes immediately after the operation the recipient would show evidence of shock, characterized by clonic and tonic muscular spasms. Complete recovery usually occurred in 3 to 4 minutes. In one instance death ensued and in 2 other animals permanent paralysis of the hind limbs resulted. 20 cc. of blood similarly collected in citrate were separated by centrifugation into plasma and cells. An equal amount without citrate was allowed to clot and the serum collected. Sterile

TABLE II.

Passive Protection of Rabbits against Pneumococcus Infection by Transfusion of Blood, Plasma, Cells, and Serum of Rabbits Immunized with R Pneumococci.

1. Protocol of Course of Bacteremia in Anti-R Donor and Recipients.

Time of blood culture	Number of colonies per unit of blood								
	Anti-R donor	Anti-R recipients				Normal donor	Normal recipients		Control 0.0001 cc. Type III
		Whole blood	Plasma	Serum	Cells		Whole blood	Serum	
2 hrs.	1	42	86	93	113	∞	20	1000	
6 hrs.	13	5	0	40	13	∞	2	816	
10 hrs.	1	9	15	62	142	∞	23	∞	
20 hrs.	58	15	7	12	518	D	148	∞	
24 hrs.	142	1	2	38	∞		272	D	
30 hrs.	32	0	29	116	∞		522		
48 hrs.	1	1	1	25	∞		∞		
72 hrs.	0	1	1	3	∞		D		D
96 hrs.	4	0	12	14	D				
5 days	0	0	1	8					
6 days	0	0	8	1					
9 days	0	0	0	0					
11 days	0*	0*	0*	0*					

0.1 cc. of rabbit virulent strain of Type III used as infecting dose.
D indicates death of the animal.
S indicates survival of the animal.
Numerals represent number of colonies per unit of blood.

precautions were observed throughout the procedure. 20 cc. of whole blood, or its equivalent in plasma, cells, or serum were then injected intravenously into normal rabbits. 24 hours later these rabbits were infected intravenously with 0.1 cc. of a rabbit virulent strain of Type III. Normal rabbits which had received comparable amounts of whole blood or serum from other normals were similarly infected. Other normal rabbits without preliminary treatment were infected with the maximal test dose and the minimal lethal amount of culture.

From Table II it may be seen that whole blood, plasma, and serum from resistant rabbits afforded protection against 1000 lethal doses of Type III pneumococcus, whereas blood cells alone were inadequate. Controls receiving normal blood or serum were unprotected. Tabulation of the number of organisms in the blood cultures reveals the fact that the resistant rabbits continued to have pneumococci in varying numbers in the blood stream from 3 to 6 days before permanent sterility was attained. Rabbits receiving normal blood, on the other hand, although possessing a slight initial capacity to reduce the number of circulating bacteria were unable to cope with the subsequent rapid increase, and died of an overwhelming septicemia.

Repetitions of protection experiments of this character using virulent Type I pneumococci instead of Type III gave results equally definite, indicating that, as in the case of active immunity, passive protection of rabbits is not limited as to type of infecting pneumococcus.

From these experiments it is established that resistance induced in rabbits by immunization with R pneumococci can be passively transferred to normal rabbits. In titering the amount of blood necessary to confer passive protection it was found that 15 to 20 cc. were necessary against doses of culture as high as those constantly employed, *i.e.* 1000 lethal doses of Type III or 10,000 to 100,000 of Type I. This quantity of blood was regularly used in all subsequent experiments. In Table III the total number of transfusions and the results are recorded. It may be seen that, with the amount of blood transfused and the dosage of culture kept constant, the time elapsing between transfusion and injection of organisms has been varied from 1 hour to 21 days. Of 5 rabbits infected within 5 hours of the time of transfusion 4 died and 1 survived. Twenty-three animals were infected 1 to 7 days after transfusion and of these 18 recovered. One rabbit infected 14 days after transfusion, survived, and of 5 in which an interval of 3 weeks elapsed, 3 recovered.

With the use of 8 to 10 cc. of serum, an amount comparable to that contained in 15 to 20 cc. of whole blood, protection was demonstrable but the results were somewhat less striking. In Table III the results of protection tests by the use of serum are presented. Of 5 rabbits infected 1 to 5 hours after serum administration, 4 survived. With an interval of 1 to 7 days, out of 9 animals tested, 4 survived; in 3 in-

TABLE III.

Passive Protection of Rabbits against Pneumococcus Infection by Transfusion of Blood and Serum of Rabbits Immunized with R Pneumococci.

2. Summary of Results of Passive Protection Tests in Rabbits.

Rabbit No.	Amount of blood	Time interval	Infection with	Result
	cc.			
1	20	1 hr.	0.1 cc. Type I	D 6 days
2	20	2 hrs.	0.1 " " "	S
3	16	4 "	0.5 " " III	D 4 days
4	15	4 "	0.5 " " "	" 4 "
5	15	5 "	0.5 " " "	" 4 "
6	20	24 "	0.1 " " I	" 4 "
7	15	24 "	0.1 " " "	S
8	20	24 "	0.5 " " III	"
9	20	24 "	0.2 " " "	"
10	20	24 "	0.2 " " "	"
11	20	3 days	0.1 " " I	"
12	20	3 "	0.1 " " "	"
13	20	3 "	0.1 " " "	D 4 days
14	20	3 "	0.01 " " "	S
15	20	3 "	0.01 " " "	"
16	20	3 "	0.5 " " III	"
17	15	4 "	0.1 " " I	D 4 days
18	15	4 "	0.1 " " "	S
19	15	4 "	0.01 " " "	"
20	20	7 "	0.01 " " "	"
21	20	7 "	0.01 " " "	"
22	20	7 "	0.1 " " "	"
23	20	7 "	0.1 " " "	D 4 days
24	17	7 "	0.1 " " "	S
25	15	7 "	0.4 " " III	"
26	15	7 "	0.5 " " "	"
27	15	7 "	0.5 " " "	D 8 days
28	20	7 "	0.1 " " "	S
29	20	14 "	0.01 " " I	"
30	20	21 "	0.01 " " "	"
31	20	21 "	0.01 " " "	"
32	20	21 "	0.01 " " "	"
33	15	21 "	0.01 " " "	"
34	20	21 "	0.01 " " "	"

D indicates death of the animal.
S indicates survival of the animal.

TABLE III—*Concluded.*

Rabbit No.	Amount of serum	Time interval	Infection with	Result
	cc.			
35	10	1 hr.	0.1 cc. Type I	S
36	10	1 "	0.1 " " "	"
37	10	2 hrs.	0.1 " " "	"
38	10	4 "	0.5 " " III	D 7 days
39	10	4 "	0.5 " " "	S
40	10	24 "	0.1 " " I	D 10 days
41	10	24 "	0.5 " " III	" 3 "
42	8	24 "	0.5 " " "	" 7 "
43	10	24 "	0.1 " " I	S
44	12	3 days	0.1 " " III	"
45	8	7 "	0.4 " " "	"
46	8	7 "	0.5 " " "	D 7 days
47	10	7 "	0.01 " " I	" 2 "
48	10	7 "	0.01 " " "	S
49	10	14 "	0.1 " " "	D 4 days
50	10	21 "	0.1 " " "	" 4 "
51	10	21 "	0.1 " " "	" 2 "

stances where the time interval was longer than 7 days, none of the animals recovered.

In all of the experiments controls were used consisting of rabbits which received quantities of normal whole bood or serum equal to the amount of immune blood or serum transferred. Altogether 20 control rabbits were transfused with normal whole blood and 10 received injections of normal serum. They all died of pneumococcus septicemia.

Differences in the effectiveness of passive protection depending on the time elapsing between administration of serum or blood and the injection of the infecting organism may be noted in Table III. In those instances where serum was employed, protection appeared to be most effective, if only a few hours elapsed before the injection of organisms. On the other hand, when whole blood was transfused, protection was less striking when the interval was short than when the animals were permitted to rest 24 hours or longer before infection. The duration of the protection conferred by whole blood is evidenced by animals which survived pneumococcus infection 3 weeks after transfusion.

No definite conclusions can be drawn at the present time from the results concerning the time intervals employed in passive protection tests. The significant fact is that the circulating blood of rabbits immunized with R pneumococci possesses active prinicples which, when transferred to normal rabbits, confer upon the recipients protection against infection with virulent pneumococci.

TABLE IV.

Comparison of Passive Protection of Mice and Rabbits by Serum of Rabbits Immunized with R_2 Pneumococci.

Anti-R rabbit serum		Amount of culture Type I	Results	
			Rabbits	Mice
cc.		*cc.*		
10	*Interval of 2 hrs.	0.1	D 24 hrs.	
10		0.01	S	
10		0.01	S	
10		0.01	S	
0.5		0.00001		D 24 hrs.
0.5		0.00001		D 24 hrs.
0.5		0.000001		D 36 hrs.
0.5		0.000001		D 36 hrs.
0.5		0.000001		D 40 hrs.
		Controls		
None		0.000001	D 60 hrs.	D 24 hrs.
None		0.000001		D 24 hrs.
None		0.000001		D 30 hrs.

D indicates death and figures the number of hours before death of animal.

* 2 hours following intraperitoneal injection of serum all animals were infected intraperitoneally with virulent culture in amounts indicated.

In contrast to the effective protection of rabbits just described, attempts to protect mice by the use of the same sera have been entirely negative. From Table IV it may be seen that serum which protected rabbits against 0.01 cc. did not protect mice against even 0.000001 cc. of culture. This was true in spite of the fact that the mice received a much larger amount of serum per unit of body weight than did the rabbits. Repeated attempts to protect mice with resistant rabbit's serum have failed regardless of whether the infecting organisms were

introduced simultaneously with serum or after intervals of 2, 6, 12, or 24 hours. The whole citrated blood of rabbits has been similarly tested and found to be without effect in mice.

It may be concluded, then, that the serum or whole blood of rabbits immunized by repeated injections of R pneumococci, although able to afford protection to normal animals of the same species, is incapable, under comparable conditions, of conferring protection upon animals of a foreign species—*i.e.* mice. These results are in striking contrast to those obtained with antipneumococcus sera which possess a high content of type-specific antibodies.

DISCUSSION.

The experiments recorded in the present communication demonstrate that a considerable degree of active immunity against Type I and Type II pneumococci may be stimulated in rabbits by repeated injections of R pneumococci. This form of resistance, elicited by R organisms which are devoid of type specificity, is effective in the absence of demonstrable *type-specific* agglutinins, precipitins, and antibodies passively protective for mice. In a preceding paper (1) it was reported that rabbits similarly treated are resistant to virulent Type III organisms; the present results with Types I and II establish the fact that immunity induced in rabbits by R strains is sufficiently broad to be effective against infection with each of the three specific types of pneumococcus. The Type III infections as previously pointed out, were characterized by a bacteremia which ran a prolonged course during which the number of circulating bacteria varied from time to time but eventually disappeared. The Types I and II infections encountered in the present experiments behave similarly. These facts are suggestive that in this form of immunity the mode of recovery from infection involves the same mechanism, or different mechanisms acting in a similar manner, against each type of pneumococcus. Furthermore, these results strongly imply that resistance under these conditions is dependent either upon other factors than those concerned in type-specific immunity, or upon the same factors operative in a different manner.

Although the majority of previous investigators have found that active immunity against pneumococcus infection is type-specific, the ex-

perimental conditions, either as to the species of animal or the method of immunization employed, have differed from those reported in this paper. Cecil and Blake (6) observed that in monkeys vaccination with living cultures of Type I conferred a certain amount of cross-immunity, the degree of effectiveness being subject to variation. The immunizing dose in their experiments consisted of *one* subcutaneous injection of either 0.001 cc. of virulent or 1 to 2 cc. of avirulent organisms. Wright (7) found that *one* preliminary intravenous injection of S pneumococci produced active immunity in rabbits effective only against homologous organisms. Barach (8) employing mice gave *one* intraperitoneal injection of S organisms and obtained strict type-specific immunity.

In the experiments here reported the active resistance which was stimulated by immunization with R pneumococci and which was found to be effective against all the fixed types, was obtained by a more prolonged series of injections. The process of immunization comprised 18 intravenous injections carried out over a period of 6 weeks according to the method described by Cole and Moore (2). Although the degree of immunization necessary to incite non-type-specific immunity has not been determined, it has been found that one injection of from 5 cc. to 25 cc. of R culture is insufficient.

The purpose of these experiments has been an attempt to understand the factors underlying the resistance. Since this form of cross-immunity can be induced by pneumococci devoid of type-specific properties, it seems highly probable that a mechanism of a different order from that involved in type-specific resistance is implicated. Work is in progress at the present to define more clearly the points of similarity and difference between these two forms of acquired resistance.

Having determined the presence of active immunity in rabbits previously treated with R cells, consideration has been given to passive immunity. Since one of the chief characteristics of type-specific immunity is the passive protection afforded animals of any species by an immune serum of the homologous type a study of the possibility of passively transferring this non-specific resistance has been carried out. It was found that whole blood or serum of R immunized rabbits protected normal rabbits against infection with virulent Type I and

Type III pneumococci.* As a rule, blood in 15 to 20 cc. amounts was found to afford a more solid resistance against a given infecting dose than the equivalent amount of serum.

Under the experimental conditions described the most striking results have been obtained when an interval of time elapsed between the transfusion and the injection of organisms. The exact significance of these relations has not as yet been sufficiently studied to justify final conclusions. However, it can be stated at the present time, that the immunity elicited by repeated injections of R pneumococci in rabbits can be passively transferred by the circulating blood to normal rabbits. This is evidence that there is present in the circulation of resistant rabbits either protective substances in an active state or something which stimulates the mechanism of resistance in the transfused animal.

Attempts to confer passive protection on mice under similar conditions have been uniformly negative. This failure is in sharp contrast to the positive results always obtained in mice with type-specific sera, and is further evidence of a difference in the mechanism involved in each instance.

SUMMARY.

1. Rabbits, vaccinated by repeated intravenous injections of suspensions of heat-killed R pneumococci, acquire a marked degree of active immunity to infection with the virulent S forms of Pneumococcus Types I and II. Previously (1) it was shown that the immunization of rabbits with R cells induces active resistance to Type III infection. This immunity is effective when the infecting organisms are injected either intravenously, intraperitoneally, or intradermally.

2. Whole citrated blood or serum of rabbits immunized with R pneumococci, under the experimental conditions described, is capable of passively protecting normal rabbits against Type I and Type III infection. Whole blood appears to be more effective than an equivalent amount of serum.

3. Passive protection of mice by the use of whole blood or serum of

* Resistance to Type II was not tested by reason of the fact that an R strain originally derived from Type II S culture was used for immunization. It seemed desirable to minimize the possible participation of type-specific substances.

the immune rabbits has been entirely ineffectual. This is in striking contrast to the results obtained with type-specific immune serum.

4. This form of acquired resistance to pneumococcus infection, elicited by R organisms which are devoid of type specificity, and exemplified in animals whose sera possess no demonstrable type-specific antibodies, has many characteristics strongly suggesting that the underlying mechanism differs from that concerned in type-specific immunity.

CONCLUSION.

A broad immunity against infection with virulent S pneumococci (Types I, II, and III) can be induced in rabbits by vaccination with the degraded R strains of pneumococcus. This form of active resistance is effective in the absence of demonstrable type-specific antibodies, and may be passively transferred to normal rabbits by the blood of the immunized animal.

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SUPPRESSION OF THE FIRST ATTACK WITH SUBSEQUENT RELAPSE: AN IMMUNE PHENOMENON IN EXPERIMENTAL RELAPSING FEVER.

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A series of experiments has already been reported (1) in which it was found that Chinese squirrels and chipmunks, when inoculated intraperitoneally with *Spironema recurrentis*, would show many spirochetes in the blood for a period of 1 to 5 days, and would recover without a relapse; but if the spleen were removed one or two relapses would occur. In a subsequent paper (2), the development of six distinct strains of spirochetes as the result of relapses was reported, and it was shown that strains which appeared in alternate attacks were closely related to one another, while strains appearing in consecutive attacks were immunologically quite different from each other.

During the course of the latter experiments a phenomenon was encountered, of which I have seen no report in the literature on relapsing fever. This phenomenon was the suppression of the first attack in animals which were reinoculated with a strain of spirochetes which had recently been present in the blood, and the subsequent occurrence of a relapse after the interval which usually occurred between attacks. That this delayed attack was a relapse and not a true first attack with a long incubation period, was proven by the fact that the spirochetes causing it were shown to belong to a different strain than the strain inoculated. The phenomenon was met with five times during the course of the experiments, as shown by the following protocols.

PROTOCOLS.

Observation 1.—A squirrel whose spleen had been removed 7 days previously, was inoculated with Strain I spirochetes. The tail blood was positive for the 4 succeed-

ing days, but no relapse occurred. After this attack the blood serum showed the presence of agglutinins only for Strain I. 32 days later the squirrel was reinoculated with Strain I. For 6 days thereafter no spirochetes appeared in the tail blood; but on the 7th day they appeared, and continued to be present in greatly increasing numbers for 4 days. After this attack the blood serum con-

TABLE I.

Course of Infection in Squirrel of Observation 1.

Date	Strain inoculated	Attacks			Negative intervals, duration	Titer of serum during interval for strain					Strain causing attack
		No.	Day	Tail blood		I	II	III	IV	V	
					days						
IV/1/27	I	1	1	+	32	1280	0	0	0	—	I
			2	++							
			3	+++							
			4	++++							
V/7/27	I			Neg.	6						I
		2	1	+	7	100+	100+	0	0	0	II
			2	++							
			3	++++							
			4	++++							
		3	1	+	6	100+	100+	100+	0	0	III
			2	+							
			3	+++							
			4	+++							
			5	+++							
		4	1	+	20						V*
			2	++							
			3	++							
			4	+							

* Origin of Strain V. Proved by agglutination tests not to belong to Strain I, II, III or IV.

tained agglutinins for both Strain I and Strain II, indicating that the spirochetes causing the delayed attack were not the strain inoculated but belonged to Strain II, the strain which usually followed Strain I in the first relapse. After another interval of 7 days another relapse occurred, which caused the development of additional agglutinins for Strain III. Another interval of 6 days then occurred, followed by another relapse. The spirochetes of this relapse were tested against

monovalent immune sera and were found to belong to Strain V (see Meleney (2) for details of strains). This squirrel, therefore, theoretically had four attacks following its reinoculation, but the first attack was suppressed because the animal was already immune to the strain of spirochetes inoculated. The record of this squirrel is summarized in Table I.

Observation 2.—A chipmunk was inoculated with Strain I spirochetes without previous splenectomy. Spirochetes were present in the tail blood on the 3 succeeding days. No relapse occurred. The blood serum after this attack contained agglutinins for Strain I but not for Strain II. Other strains were not tested. 12 days after the close of the attack the spleen was removed and 5 days after that the animal was reinoculated with Strain I spirochetes. The tail blood was negative for the next 5 days, after which an attack occurred which lasted 4 days. After this attack the animal's blood serum contained agglutinins for both Strain I and Strain II, indicating that the attack had been due not to the Strain I spirochetes with which it had been reinoculated, but to Strain II.

Observation 3.—A second chipmunk was inoculated with Strain I spirochetes without previous splenectomy. The tail blood was positive for spirochetes on the 3 succeeding days. No relapse occurred. The spleen was removed 16 days later, and 14 days after splenectomy the animal was reinoculated with Strain I spirochetes. The tail blood remained negative for 5 days, after which an attack ensued with spirochetes in the blood for 5 days. After this the animal's serum was found to contain agglutinins for Strain II, indicating that the attack had been due to that strain rather than to Strain I, with which the animal had been reinoculated. After another interval of 8 days a second relapse occurred lasting 2 days.

Observation 4.—A squirrel was inoculated on the day following splenectomy with Strain II spirochetes. After an incubation period of 3 days spirochetes appeared in the blood for 2 days. The attack was followed by a negative interval of 7 days and a relapse lasting 3 days. There was no second relapse. The animal's serum after the relapse contained agglutinins for Strain II, Strain I and Strain III.[1] After waiting 20 days from the close of the relapse, this squirrel was reinoculated with Strain III spirochetes. A negative interval of 6 days ensued, and then spirochetes appeared in the blood for 3 days. After this attack the animal's serum contained additional agglutinins for Strain V, indicating that the attack had been due to that strain rather than to Strain III, with which it had been reinoculated. After another interval of 4 days a relapse occurred in which the spirochetes were proven by agglutination tests to belong to Strain VI.

Observation 5.—This squirrel was inoculated three different times. The first inoculation was made with Strain I on the day following splenectomy. A single attack followed which lasted 5 days. After this attack the animal's serum contained agglutinins only for Strain I. After waiting 23 days it was reinoculated with Strain I and the tail blood contained spirochetes for the 3 following days.

[1] It was frequently found that, after a first relapse in an animal inoculated with Strain II, agglutinins for both Strain I and Strain III were present.

Thus the first reinoculation was not followed by suppression of the first attack as in the other four observations. After an interval of 7 days a relapse occurred lasting 3 days, and after another interval of 9 days a second relapse occurred lasting 3 days. The spirochetes of this second relapse were proven by agglutination tests with monovalent sera to belong to Strain III, but after this relapse the animal's serum contained agglutinins not only for Strains I, II and III, but also for Strain IV. 16 days later it was reinoculated with Strain IV spirochetes. The tail blood was negative for 8 days, after which spirochetes appeared for 3 days, and were found by agglutination tests to belong to Strain V.

COMMENT.

It is apparent from the above protocols that the reason that the "first attack" failed to appear following the reinoculation in these animals, was that they were immune to the strain of spirochetes with which they were reinoculated. The spirochetes which were injected into the immune animal found themselves in an unfavorable environment. Most of them were destroyed, but a few survived because they were able to adjust themselves to the environment. In other words, the course of the disease in these animals from the time of the reinoculation was the same as that in animals which have just reached the crisis of their first attack, when the blood still contains spirochetes and the immune bodies against those spirochetes have already begun to appear. In such animals the spirochetes *in the blood stream* are rapidly destroyed, causing the first attack to stop abruptly. In the reinoculated animals the first attack is entirely suppressed by the destruction of the spirochetes *in the peritoneal cavity*. In both types of animals a few spirochetes survive and undergo a biological change into a new strain. About 6 days is required for the spirochetes of the new strain to become numerous enough to be found in the peripheral blood. This is the negative interval, and it is followed by the relapse due to the new strain of spirochetes.

This phenomenon is an additional demonstration of the immunity to a single strain of *S. recurrentis* which is conferred by an attack due to that strain. It also emphasizes the limitation of such immunity to the strain or strains which have been present in preceding attacks or to closely related strains.

In some papers on experimental relapsing fever (3), long incubation periods are described in mice which have been reinoculated with the

same strain as that used originally. It may be that these cases are examples of the phenomenon here described. (See Kudicke and Feldt, Tables 2, 5 and 6.) The present observations emphasize the importance of determining the identity of the strain of relapsing fever spirochetes which appears in any attack other than one immediately following inoculation, if reliable conclusions are to be drawn as to the immunological reactions which take place between this parasite and its host. The strain of each relapse is a strain which was not present in the animal's blood in a previous attack. In order to distinguish between a prolonged incubation period and a suppressed first attack followed by a relapse, it is necessary either to test the spirochetes causing the attack, against monovalent immune sera, or to determine, after the close of the attack, what additional agglutinins have been added to the animal's serum as a result of the attack.

SUMMARY.

In five splenectomized squirrels and chipmunks which were reinoculated with a strain of *Spironema recurrentis* which had previously been present in their blood, the first attack was entirely suppressed because the animals were immune to the strain of spirochetes inoculated; but after the interval which usually occurred between attacks, a relapse ensued, in which the strain of spirochetes present in the blood was different from the strain inoculated.

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OBSERVATIONS ON FLAGELLAR AND SOMATIC AGGLUTINATION.

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In a recent review Hadley (1) presented a critical discussion of the flagellar antigen concept as originally proposed by Smith and Reagh (2) to account for the difference in agglutination between motile and non-motile strains of the hog cholera bacillus. He questioned the validity of the hypothesis on the ground that the particular order of serologic reaction was not limited to bacterial species characterized by the possession of flagella. He suggested that such serologic differences might eventually come to be studied in terms of the presence or absence of certain specific soluble substances wherever their point of origin in the bacterial cell. Some work done by the writer on the agglutinability of deflagellated motile bacteria seemed to have a bearing on the latter suggestion. The work was extended somewhat and is discussed in the present paper.

The initial work of Smith and Reagh (2) was concerned with the agglutinative affinities of two strains of the hog cholera bacillus, one motile, the other a non-motile variant. A "motile" antiserum agglutinated the motile strain in high dilution with a floccular type of clump. Microscopically the clumped bacilli were separated by narrow spaces. It agglutinated the non-motile strain in a lower dilution with granular clumps in which the bacteria were not separated by any appreciable space. A "non-motile" antiserum agglutinated both strains in relatively low dilution with granular clumping. The non-motile strain removed the granulating but not the flocculating agglutinin from "motile" antiserum. The motile strain reduced the granulating agglutinin of the "non-motile" serum. The writers concluded that the agglutinins for the flagella and for the body of the bacilli, at least so far as the large group of pathogenic colon derivatives were concerned, were distinct, not mutally interacting substances.

These observations were subsequently extended by other workers among whom the following may be mentioned. Beyer and Reagh (3), working under Smith,

showed that flagellar antigen and somatic agglutinin were heat-labile at 70°C. while somatic antigen and flagellar agglutinin were heat-stable. Orcutt (4) working with the hog cholera bacillus and Balteanu (5) with the vibrio comma demonstrated the agglutinative and antigenic properties of pure flagellar suspensions. Yokota (6) carried out similar studies on flagellar suspensions from *B. typhosus* and also on the bacteria deflagellated by shaking. Goyle (7) compared the agglutinative relationships of heated and untreated suspensions of *B. typhosus* and *B. enteritidis* and their variants. Arkwright (8) described the microscopic findings with floccular and granular agglutination.

The multiple antigen hypothesis as related to bacteria has also been criticized by Tulloch (9) in an extended series of papers. He pointed out certain irregularities which cannot be cited here.

Direct Agglutination of Whole, Shaken and Heated Antigens by "Whole" Antiserum.

Two species of Salmonella designated *B. paratyphi* Types I and II were employed in the present work.

The strains used were originally cultivated from spleen tissue. They were isolated during the course of a natural epidemic in a guinea pig population (10). The final cultures represented the growth from single colonies replated three times on agar. They were normal smooth strains as to their growth in broth and their colony formation on agar, were actively motile and gave a characteristic agglutination with diagnostic antiserums then in use.

Fresh antiserums against the whole bacteria were prepared in rabbits.

The growth from 18 hour agar cultures was removed with physiological salt solution, centrifuged, the packed bacteria washed once, resuspended in 0.2 per cent formalinized saline and heated to 56°C. for 1 hour. Five intraperitoneal injections at 3 day intervals were given. The rabbits were previously tested for normal or immune agglutinins active for the particular antigens. The two type antiserums, termed "whole," were tested against whole, heated and shaken suspensions of both types of *B. paratyphi*. The suspensions were prepared as follows: The growth from a Blake bottle was removed with 10 cc. of saline, washed once, resuspended in saline and divided into three portions. One was untreated. One was heated to 100°C. for 30 minutes in a water bath, washed once after centrifuging and resuspended in saline. One was shaken for 1 hour in a mechanical shaker, washed twice after centrifuging and resuspended in saline. The three suspensions were finally standardized to equal opacity, 2.4 on the Gates scale. In testing, 0.5 cc. amounts of diluted serum and suspension were mixed. The final dilutions were doubled at each interval from 1:100 through 1:51,200. The

tubes were incubated at 37°C. for 3 hours and read after overnight refrigeration. The limits of agglutination with each serum against the six antigens are given in Table I. The limit of agglutination is defined as the highest dilution showing any macroscopic evidence of clumping after approximately 24 hours.

The agglutination of the several antigens with reference to the character of the sediment and the limiting dilution was much the same with both antiserums. The whole antigen was agglutinated in high dilution by its homologous serum. The heated and shaken antigens were agglutinated equally through a much lower limit. With the

TABLE I.

Direct Agglutination of Whole, Shaken and Heated Antigens with Type I and Type II Antiserums.

Serum	Antigen	Limiting dilution	Type of agglutination
Type I "whole"	Type I whole	1:25,600	Mixed
	" " shaken	1:3,200	Granular
	" " heated	1:3,200	"
	" II whole	1:800	Atypical granular
	" " shaken	1:800	Granular
	" " heated	1:800	"
Type II "whole"	" II whole	1:25,600	Mixed
	" " shaken	1:3,200	Granular
	" " heated	1:3,200	"
	" I whole	1:200	Atypical granular
	" "shaken	1:200	Granular
	" " heated	1:200	"

antiserums of opposite type the three antigens were agglutinated equally in still lower dilution. The reaction with the homologous whole antigen was predominantly floccular throughout with an admixture of granular clumps in the lower dilutions. The reaction was rapid with the formation of coarse clumps which on settling formed a light feathery sediment. In the lower dilutions the supernatants were clear or nearly so. Microscopically the sediment was composed mainly of large, poorly defined clumps with an open work appearance. With the lower dilutions there were also smaller compact clumps which were more sharply defined. The type of agglutination with the ho-

mologous heated and shaken antigens was granular throughout. The reaction was retarded. The sediment varied from an irregular, wrinkled, compact disc in the lower dilutions to a granular mold fitting the rounded portion of the tube in the higher dilutions. The clumps were not easily broken up on shaking. The supernatants tended to be clear up to the last two dilutions with a graded sediment. With the antigens of the opposite type the agglutination was granular. The macroscopic picture with the heated and shaken antigens was identical with that described above. The character of the sediment with the whole antigens was somewhat different. The Type II antigen formed a compact, even, button-like sediment with a practically clear supernatant. The Type I antigen formed a sediment closely approaching the typical granular form except that the clumps were larger and not as closely packed. Microscopically the clumps were of the granular type in both cases.

Absorption of "Whole" Antiserum with Whole, Shaken and Heated Antigens.

The two "whole" antiserums were absorbed with the three homologous antigens.

In absorbing 0.25 cc. of packed bacteria, 0.1 cc. of serum and 2.4 cc. of 0.2 per cent formalinized saline were employed, an absorbing dose and a serum dilution of approximately 1:10 and 1:25 respectively. The absorbing antigens were prepared as previously described and sedimented for 1 hour in graduated centrifuge tubes to a constant volume. The final antigen-serum mixtures were incubated at 37°C. for 3 hours with frequent shakings. After overnight refrigeration they were centrifuged and the clear supernatants tested. The dilution series previously described was employed. Unabsorbed serum similarly diluted with formalinized saline was subjected to the same incubation and tested with the same antigen suspension. The results were identical with those given in Table I. The limiting agglutination of the three antigens by the absorbed serums is given in Table II.

After absorption with whole antigen both serums continued to give a floccular reaction with the homologous antigen in very low dilution. There was no reaction with the shaken and heated antigens in the lowest dilution employed. Absorbed with shaken and heated antigens both serums agglutinated the homologous whole antigen through dilution 1:12,800, one dilution removed from the limit of agglutina-

tion with the unabsorbed serum. The type of clumping was purely floccular in all cases. The supernatants showed a diffuse turbidity which was graded. There was no agglutination of the shaken and heated antigens in the lowest dilution. The removal of a considerable amount of flocculating agglutinin by the treated antigens was indi-

TABLE II.

Agglutination Limits with Type I and Type II Antiserums after Absorption with Whole, Shaken and Heated Antigens.

Serum	Absorbing antigen	Agglutination after absorption	Antigen agglutinated
Type I whole	Type I whole	1:400	Whole
		<1:100	Shaken
		<1:100	Heated
	Type I shaken	1:12,800	Whole
		<1:100	Shaken
		<1:100	Heated
	Type I heated	1:12,800	Whole
		<1:100	Shaken
		<1:100	Heated
Type II whole	Type II whole	1:200	Whole
		<1:100	Shaken
		<1:100	Heated
	Type II shaken	1:12,800	Whole
		<1:100	Shaken
		<1:100	Heated
	Type II heated	1:12,800	Whole
		<1:100	Shaken
		<1:100	Heated

cated. Further evidence bearing on the absorption of flocculating agglutinin by the treated antigens will be presented in another paper.

Agglutination with Shaken and Heated Antiserums before and after Absorption.

The immunizing properties of the shaken and heated suspensions of the motile bacteria were determined.

The suspensions were prepared as described. The treated and washed bacteria were sedimented in the centrifuge, resuspended in 1 per cent formalinized saline and kept for 3 days in the refrigerator. They were resedimented, washed twice and resuspended in 0.2 per cent formalinized saline. It was necessary to kill the shaken bacteria and the above method was chosen in preference to heat at 56–58°C. on successive days. To keep the suspensions uniform both were subjected to the same conditions although the heated suspension (100°C. for 30 minutes) was obviously inactivated. Subcultures made on consecutive days were sterile. A rabbit was immunized intraperitoneally with each suspension. Five injections at 3 day intervals were given with the Type I antigens, four injections with the Type II. 7 days after the last injection the animals were bled and killed. At autopsy no focal lesions were detected in any of the usual sites: spleen, liver or lymphoid tissue of the intestinal tract. There were traces of exudate on one or another of the visceral organs. Walled-off accumulations of purulent material along the cecum were commonly observed. Cultures from spleen, liver, purulent material and cecal feces failed to show *B. paratyphi*. The agglutinin content of the several antiserums was determined by direct agglutination and by absorption employing whole, shaken and heated antigens of the same type. The limits of agglutination of the shaken and heated antiserums before and after absorption are given in Table III.

The shaken and heated antiserums agglutinated the three homologous antigens equally. The limiting dilutions were higher with the Type I serums as might be expected in view of the longer immunization. In both cases the clumping of the shaken and heated antigens was typically granular. The sediment with the whole antigens was granular microscopically. Macroscopically the sediment with each type was identical in appearance with that described previously for whole antigen agglutinated by serum of opposite type.

The absorption tests gave further evidence for the absence of flocculating agglutinin. Absorption with any one antigen removed most of the agglutinin for all three suspensions. Irregularities in the titer were noted but had no bearing on the presence or absence of flocculating agglutinin. In general the reduction of agglutinin was a little less complete than that previously noted with the whole antiserums. Although the shaken and heated antigens were able to remove some flocculating agglutinin from whole antiserum, there was no indication that they were able to produce the same upon injection in rabbits.

TABLE III.

Agglutination Limits with Shaken and Heated Serums of Both Types before and after Absorption.

Serum	Direct agglutination	Antigen agglutinated	Absorbing antigen	Agglutination after absorption	Antigen agglutinated
Type I shaken	1:6,400	Type I whole	Type I whole	1:200	Type I whole
				1:200	" " shaken
				1:200	" " heated
	1:6,400	Type I shaken	Type I shaken	1:200	" " whole
				1:200	" " shaken
				1:200	" " heated
	1:6,400	Type I heated	Type I heated	1:200	" " whole
				1:200	" " shaken
				1:200	" " heated
Type I heated	1:6,400	Type I whole	Type I whole	1:200	Type I whole
				1:200	" " shaken
				1:100	" " heated
	1:6,400	Type I shaken	Type I shaken	1:200	" " whole
				1:200	" " shaken
				1:200	" " heated
	1:6,400	Type I heated	Type I heated	1:200	" " whole
				1:200	" " shaken
				1:100	" " heated
Type II shaken	1:3,200	Type II whole	Type II whole	<1:100	Type II whole
				<1:100	" " shaken
				<1:100	" " heated
	1:3,200	Type II shaken	Type II shaken	1:100	" " whole
				1:100	" " shaken
				1:100	" " heated
	1:3,200	Type II heated	Type II heated	1:100	" " whole
				1:100	" " shaken
				1:100	" " heated
Type II heated	1:3,200	Type II whole	Type II whole	1:200	Type II whole
				1:200	" " shaken
				1:200	" " heated
	1:3,200	Type II shaken	Type II shaken	1:200	" " whole
				1:200	" " shaken
				1:200	" " heated
	1:3,200	Type II heated	Type II heated	1:100	" " whole
				1:100	" " shaken
				1:100	" " heated

Agglutination of Untreated, Shaken and Heated Suspensions of a Non-Motile Bacterium.

The agglutination tests were repeated with a culture of *Staphylococcus aureus* and its homologous antiserum to afford a basis for comparing the agglutinative affinities of a non-motile organism. The staphylococcus was recently isolated from a skin abscess and was a normal, smooth strain. A rabbit antiserum against the whole antigen was prepared. The same procedures outlined for the motile bacteria were followed in immunization, preparation of antigens and performance of the agglutination tests. The limits of agglutination before and after absorption with whole and heated antigens are given in Table IV.

TABLE IV.

Limits of Agglutination with Staphylococcus Antiserum before and after Absorption

Serum	Direct agglutination	Antigen agglutinated	Absorbing antigen	Agglutination after absorption	Antigen agglutinated
Staphylococcus whole	1:1,600 G*	Whole	Whole	1:50 G	Whole
			"	1:50 G	Heated
	1:1,600 G	Heated	Heated	1:50 G	Whole
			"	1:50 G	Heated

* Granular clumping.

The shaken antigen showed the same limiting dilution upon direct agglutination and was not employed in absorption.

The staphylococcus antiserum agglutinated whole and heated antigens to the same titer limit. The reactions were retarded, the limiting dilutions were relatively low and the clumping was typically granular both macroscopically and microscopically. The titer of the serum, 1:1,600 after five injections, was not noticeably increased by three additional injections. The agglutination of whole and heated antigens by the "non-motile" antiserum was identical in character with that of heated and shaken antigens by the "motile" antiserums. The whole and heated staphylococcus antigens possessed approximately the same absorptive capacity for agglutinin as indicated by partial absorption. Reciprocal absorption with a "heated" staphylococcus antiserum was not made.

The Origin of Soluble Material Extracted by Heat from the Motile Bacteria.

Some additional experiments bearing on the production of soluble material from the motile bacteria may be cited. A considerable decrease was noted in the volume of packed bacteria after heating to 100°C. for 30 minutes. Goyle (7) had previously called attention to the decreased opacity of heated bacterial emulsions. The supernatants from such heated suspensions after sedimentation of the bacteria were distinctly milky in appearance and when mixed with "whole" antiserum gave a precipitate in moderate dilution. Specific soluble material precipitable with immune serum had evidently been extracted from the bacteria at the temperature employed. Happold (11) has recently described a precipitinogen present in the filtrates of steamed broth cultures of *B. aertrycke* (mutton).

Comparative tests were made on the supernatants from heated whole suspensions and heated shaken suspensions to determine the effect of the presence of flagella on the amount of extracted soluble material. Filtrates of the growth from 18 hour agar cultures of the two motile bacteria were included.

Blake bottles were inoculated with 2.5–3 cc. of 6 hour broth cultures of the bacteria and incubated at 37°C. for 18–24 hours. The growth from each bottle was removed with 5 cc. or so of saline, transferred to a graduated tube and centrifuged for 1 hour. The supernatant was withdrawn and the packed bacteria washed once with 8 cc. of saline. Both supernatants were saved and later filtered through medium Berkefeld candles. For the final suspensions 1 cc. of saline was added for each 0.1 cc. of packed bacteria and the cells resuspended. One suspension of each type was shaken for 1 hour. The packed bacteria, after centrifuging, were washed three times and finally resuspended in saline according to the same ratio. The untreated and the shaken suspensions of each type were then heated to 100°C. for 1 hour in a water bath. After centrifuging the supernatants were removed and filtered through Berkefeld filters.

The final solutions prepared according to the above methods all had a milky, opalescent appearance. There was no deposit upon standing. The culture supernatants were perfectly clear but showed a yellowish tinge. The volume of packed bacteria before heating was approximately 1 cc. with Type I, 0.7 cc. with Type II. After heating, the volumes were 0.7 cc. and 0.4 cc. respectively. The suspensions were centrifuged for 1 hour at the same speed in both cases. The culture and suspension filtrates were tested with "whole" antiserum of the same type. Only one culture filtrate was employed with the Type II *B. paratyphi*.

In testing, the antigens were diluted in series and the serum kept constant, 0.5 cc. amounts of the former and 0.1 cc. amounts of the latter being employed. The tubes were incubated at 37°C. for 1 hour, followed by overnight refrigeration. The final readings are given in Table V. The first culture filtrates and the suspension filtrates showed a ring reaction with beginning precipitation in the lowest dilutions after 1 hour. Upon standing there were decreasing amounts of a granular sediment, indicated in the table by plus signs. The highest dilutions showed a distinct clouding of the supernatant but no sediment. No bacteria were detected upon microscopic examination.

TABLE V.

Precipitin Tests with Culture Filtrates and Heated Suspension Filtrates of the Two Salmonella Types.

Antigen	Serum	Serum dilutions								
		1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280
Type I	Type I									
1st culture filtrate	whole	+	+	±	Cl.*	S.cl.*	−	−	−	−
2nd " "		S.cl.	−	−	−	−	−	−	−	−
Heated whole suspension		++	++	+	+	±	±	Cl.	S.cl.	−
" shaken "		++	++	+	+	±	±	Cl.	S.cl.	−
Type II	Type II									
1st culture filtrate	whole	+	±	±	S.cl.	−	−	−	−	−
Heated whole suspension		++	++	++	+	±	±	Cl.	S.cl.	−
" shaken "		++	++	++	+	±	±	Cl.	S.cl.	−

* Cl. = cloudy supernatant; S.cl. = slightly cloudy supernatant.

The above tests indicate the presence of small amounts of soluble precipitate material in the diluted culture filtrates of both Salmonella species. The amount was greatly reduced by one washing as indicated by the reaction with the second Type I culture filtrate. Similar material was extracted from the bacteria by heat and was present in considerably greater amount in the diluting medium. The supernatant from the heated whole bacteria reacted quantitatively the same as that from the heated shaken bacteria. The amount of soluble material present in the fluid as a result of flagellar disintegration was evidently too small to affect the titer. It may be said, too, that the

agglutinability of the bacteria was not greatly influenced by the loss of the somatic constituents extracted by heat.

DISCUSSION.

The experimental work on agglutination reported in the previous section is in no sense original. The general conformation of the observations to the early flagellar hypothesis of Smith and Reagh (2) and its extension by others is the main reason for its presentation. The observations bear out the altered agglutinability of motile bacteria following shaking and heating at 100°C. Both treatments result in deflagellation of the bacteria. With the former the flagella are mechanically broken off and subsequently removed by washing. With the latter a disintegration of the flagella occurs and the soluble products are removed upon washing. Tulloch (9) has objected to the application of heat for demonstrating separate antigens on the ground that the physical nature of the bacterial cell as a whole may undergo a change resulting in altered agglutinability. The results following deflagellation by shaking are less easily disposed of in this way. No marked change in the physical state of the cell is apparent, aside from the removal of one morphological structure, the flagellum. The shaken bacteria are viable and capable of normal growth upon transfer to media. In common with the heated bacteria, however, they are incapable, in the present case, of producing flocculating agglutinin upon injection in rabbits. Admittedly there is lack of agreement concerning the true antigenic nature of deflagellated bacteria. In some instances at least the reported ability of treated bacteria to produce flocculating agglutinin appears referable to the presence of flagellar material in the suspension used for immunization.

One exception to the flagellar hypothesis was noted with the present work. The shaken and heated suspensions were able to absorb a certain amount of flocculating agglutinin from "whole" antiserum. It is suggested that the anomalous reaction may be attributed to a non-specific adsorption of antibody. Regarding the reaction in an animal host as the more exacting criterion it appears that the motile bacteria have lost in immunizing ability with the loss of flagella. This in turn implies that the substance of the flagella embraces a specific antigen.

The objections of Hadley (1) to such an assumption have been previously noted. He intimates that serological differences similar to those noted with whole and deflagellated bacteria may be explained by the presence of soluble specific substances disregarding particular morphological elements.

A small amount of soluble material precipitable with immune serum was found present in culture medium supporting the growth of the motile bacteria. The suspensions used for the production of "whole" antiserum were carefully washed and the amount of such material reduced to a practically negligible quantity. The resulting antiserum, however, contained both granulating and flocculating agglutinin. If a soluble substance were responsible for the appearance of flocculating agglutinin the bulk of it must have been produced from the bacteria after their introduction into the animal host. The "shaken" and "heated" antiserums contained only granulating agglutinin. Hence if a soluble substance is to be regarded as the antigen leading to the production of flocculating agglutinin it seems necessary to associate it with the flagella. Orcutt (4) has shown that pure flagellar suspensions (hog cholera bacillus) give a floccular agglutination with "whole" or flagellar antiserum. If the suspension is heated to 70°C. for 30 minutes the flagella are broken up. The floccular agglutinability is lost but on animal injection the heated suspension produces flocculating agglutinin. Apparently the flagella go into solution. With our experiments such heated suspensions in moderate dilution (1:80) gave a granular precipitate with "whole" antiserum. One might regard this reaction as the analytical production of soluble specific material which was antigenic in the sense that it could still produce antibody but which because of a change in the physical state of its precursor, the flagellum, showed altered agglutinability. Injected bacteria must eventually undergo disintegration probably with the production of similar soluble materials. Viewed in this light the suggestion of Hadley seems plausible with this modification, however, that the soluble substance be limited as an antigen to the flagella.

Hadley (1) has also questioned the purity of the flagellar suspensions used by Orcutt (4) and Balteanu (5). He suggests that the heat-labile factor present in such suspensions may equally well represent

soluble substances from the bacteria themselves. We have shown that the bulk of soluble precipitable material extracted *in vitro* from motile bacteria or produced *in vivo* in smaller amounts during growth originates in that portion of the bacterial cell which as an antigen gives rise to granular agglutinin. In combination with "whole" antiserum this material gives a precipitate which is likewise granular in nature. Its presence in flagellar suspensions could not be held accountable for the floccular reaction which occurs with "whole" or flagellar antiserums.

SUMMARY.

Whole, shaken and heated suspensions of two Salmonella species were compared as to agglutinability, absorptive capacity and antigenic properties. The results were in general agreement with the flagellar antigen concept of Smith and Reagh. The removal of flagella by shaking or heating (100°C.) resulted in altered agglutinability manifested by failure to give a floccular reaction with "whole" antiserum. The deflagellated bacteria were able to absorb some flocculating agglutinin from that serum. They were unable, however, to produce flocculating agglutinin upon injection in rabbits.

Untreated, shaken and heated suspensions of a non-motile bacterium (*Staphylococcus*) showed no differences with respect to agglutinability or absorptive capacity.

Soluble precipitable material was found present in small amount in culture filtrates of the motile bacteria and in greater concentration in filtrates of heated suspensions. The bulk of the soluble material was of somatic origin and was not appreciably increased by the presence of flagella. It was possible, however, to demonstrate soluble material in heated flagellar suspensions. The relation of such soluble substances to floccular agglutination and the production of flocculating agglutinin as suggested by Hadley is discussed.

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THE REMOVAL OF AGGLUTININ FROM SENSITIZED MOTILE BACTERIA.

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The flagellar antigen hypothesis of Smith and Reagh (1) assumes that motile bacteria as antigens give rise to two distinct types of agglutinin, termed flagellar and somatic agglutinin respectively. The former is produced by the flagella and reacts with them, whether attached to the cell or free, to give a floccular agglutination. The latter is produced by the bacterial cell proper and reacts with whole or deflagellated bacteria to give a granular agglutination. The somatic antibody does not combine with flagella. Some experiments on the agglutinability of whole and deflagellated suspensions of motile bacteria were reported in a preceding paper (2). The performance of absorption tests, during the course of the work, suggested that it might be possible to demonstrate differences between the two agglutinins with respect to their removal from sensitized bacteria.

A number of methods have been employed with more or less success in attempts to remove antibody from combination with sensitized bacteria. The early work has been reviewed in some detail by Huntoon (3). In another paper Huntoon and Etris (4) have reported an extensive series of experiments designed to secure purified solutions of antibody, largely by removal from sensitized antigens. They worked with non-motile bacteria chiefly the pneumococcus but also the meningococcus and *B. dysenteriæ* Flexner. They showed that agglutinin could be removed by suspending the sensitized antigen in 10 per cent saccharose solutions or distilled water and heating to 55–60°C. for 1 hour. They were able to remove as high as 50 per cent of the combined antibody. They found that the presence of sodium chloride interfered with the removal of agglutinin but not with the removal of bactericidal and protective antibodies. A temperature of 65°C. reduced the dissociation of antibody and 70°C. almost completely prevented it. Other experiments reported by Huntoon and Etris on the removal of protective antibody from sensitized pneumococcus have no immediate bearing on the present work.

The close association of antibodies with serum globulins suggested the application of some method of globulin extraction for the removal of agglutinin. In some preliminary work it was found that heating sensitized motile bacteria to 60°C. in the presence of 5 per cent sodium chloride solution resulted in the removal of a considerable amount of one agglutinin but little or none of the other. More detailed observations on the application of the method are reported in the following experiments.

Two species of *B. paratyphi* (Types I and II) (5) of guinea pig origin were employed in the absorption and extraction tests. The serums were from rabbits immunized with inactivated suspensions of the whole bacteria.

In the absorption tests a serum dilution of approximately 1:25 and an absorbing dose of 1:10 were employed. The bacterial suspensions were freshly prepared, washed once and centrifuged to constant volume—0.25 cc. 0.1 cc. of "whole" antiserum and 2.4 cc. of physiological salt solution were added and the packed bacteria resuspended. The absorption mixtures were incubated for approximately 5 hours at 37°C. followed by overnight refrigeration. Unabsorbed serum in the same dilution was included as a control. The sensitized bacteria were sedimented in the centrifuge and the clear supernatants saved. The packed bacteria were washed four times by resuspending in 5 cc. of saline and centrifuging. After the fourth washing the packed bacteria were resuspended in 5 cc. of 5 per cent sodium chloride solution and heated to 60°C. for 1 hour in a water bath. The mixture was shaken at frequent intervals. After a final centrifuging the supernatants were withdrawn. In several instances second and third extractions were carried out in the same manner.

The agglutinin titer of the several supernatants was determined by macroscopic agglutination tests. 0.5 cc. amounts of diluted test fluid and of bacterial suspension were employed. Whole and heated suspensions were used as antigens. The former was a fresh, untreated culture, washed, and diluted to standard opacity with saline. It represented both flagellar and somatic antigens. The latter was a fresh suspension heated to 100°C. for 30 minutes, washed and similarly diluted. It represented pure somatic antigen. The tubes were incubated for 3 hours at 37°C. and read after overnight refrigeration.

The agglutination readings with unabsorbed serum, absorbed serum, four washing fluids and three extraction fluids are given in Table I. The work was done with Type II *B. paratyphi* and its homologous "whole" antiserum.

The unabsorbed serum agglutinated the whole antigen through a

TABLE I.

Agglutination of B. paratyphi Type II by "Whole" and Absorbed Serum, Washings and Extraction Fluids.

Test fluid	Antigen	Test fluid dilutions										
		2	4	8	16	32	64	128	256	512	1,024	2,048
Unabsorbed serum	W*	CM	C	C	C	++++	++++	+++	+++	++	+	−
	H	CG	C	++++	+++	++	+	±	−	−	−	−
Absorbed serum	W	++M	+	±	−	−	−	−				
	H	±G	−	−	−	−	−	−				
1st washing	W	+F	±	−	−	−	−	−				
	H	−	−	−	−	−	−	−				
2nd washing	W	±F	−	−	−	−	−	−				
	H	−	−	−	−	−	−	−				
3rd washing	W	±F	−	−	−	−	−	−				
	H	−	−	−	−	−	−	−				
4th washing	W	±F	−	−	−	−	−	−				
	H	−	−	−	−	−	−	−				
1st extraction	W	+++F	+++	+++	+++	+++	++	++	++	+	−	−
	H	−	−	−	−	−	−	−				
2nd extraction	W	+++F	+++	++	++	+	±	−				
	H	−	−	−	−	−	−	−				
3rd extraction	W	++F	+	±	−	−	−	−				
	H	−	−	−	−	−	−	−				

* M = mixed agglutination; F = floccular agglutination; G = granular agglutination; W = whole antigen; H = heated antigen.

dilution of 1:1,024, the heated antigen through 1:128. The actual titer limits of the serum were 1:25,600 and 1:3,200 respectively, since an initial dilution of 1:25 was used. With the whole antigen the clumping was predominantly floccular with an admixture of granular clumps in the lower dilutions. With the heated antigen the clumping was purely granular. After absorption with the whole antigen there was a residual agglutination in low dilution. The bulk of the two agglutinins had combined with the bacteria. The first washing fluid slightly agglutinated the whole antigen in low dilution. There was no reaction with the heated antigen. The other three washing fluids in spite of progressive dilution continued to give a slight floccular reaction with whole but not with heated antigen. The first extraction supernatant gave a reduced but perfectly definite agglutination purely floccular in type through a dilution of 1:512 with the whole antigen. For comparison with the agglutinin titer of undiluted serum the initial dilution of the absorbed serum must be considered, giving a final dilution of 1:12,800. There was no reaction with the heated antigen in any dilution. A second and third extraction gave decreasing floccular agglutination with the whole antigen but no reaction with the heated.

The persistent agglutination with the washing fluids suggested that a small amount of flocculating agglutinin might be removed at room temperature. Increasing the temperature and salt content of the suspending fluid gave a marked increase in the amount removed. The method of extraction failed to remove a detectable amount of granulating agglutinin.

The above experiment was repeated with different strains of the same bacterium and with another species of Salmonella, *B. paratyphi* Type I. The results were always much the same. The limiting dilutions of the absorbed serum, washing fluids and extraction fluids varied somewhat. In several instances the titer of the first extraction fluid was one dilution lower. Sometimes, too, there was a slight granular sediment in the lowest dilution of the heated antigen series. The substitution of suspensions deflagellated by shaking instead of by heat gave identical results. Adequate controls were included for the effect of 5 per cent sodium chloride solution on whole antigen in the absence of serum and on heated antigen in the presence of

serum. There was no indication of spontaneous agglutination or of inhibition of granular clumping.

The extraction experiment was repeated with a sensitized nonmotile bacterium. A strain of *Staphylococcus aureus* and its homologous antiserum were employed. The results are given in Table II. The unabsorbed serum agglutinated both antigens to the same titer limit, 1:1,600, with a granular type of clump. There was a slight residual agglutination after absorption with the untreated suspension.

TABLE II.

Agglutination of Staphylococcus by Unabsorbed and Absorbed Serum, Washing Fluid and Extraction Fluid.

Test fluid	Antigen	Test fluid dilutions							
		2	4	8	16	32	64	128	256
Unabsorbed serum	W*	CG	++++	++++	+++	++	+	−	−
	H	CG	++++	+++	++	+	±	−	−
Absorbed serum	W	±G	−	−	−	−	−	−	−
	H	±G	−	−	−	−	−	−	−
1st washing	W	−	−	−	−	−	−	−	−
	H	−	−	−	−	−	−	−	−
1st extraction	W	−	−	−	−	−	−	−	−
	H	−	−	−	−	−	−	−	−

* W = unheated antigen; H = heated antigen; G = granular agglutination.

The washing and extraction fluids failed to agglutinate either antigen in any dilution.

The extraction method was also applied to sensitized suspensions of the two motile bacteria previously deflagellated by shaking and heating respectively. The removal of some flocculating agglutinin from "whole" antiserum by such suspensions was noted in a preceding paper (2). The absorptions together with a control of unabsorbed serum were carried out in 0.2 per cent formalinized saline. Otherwise the method of procedure was identical with that previously outlined. The results for *B. paratyphi* Type II and its homologous "whole" antiserum are given in Table III.

TABLE III.

Agglutination of Shaken and Heated Suspensions of B. paratyphi Type II by "Whole" and Absorbed Serum, Washing and Extraction Fluids.

Test fluid	Antigen	Test fluid dilutions										
		2	4	8	16	32	64	128	256	512	1,024	2,048
Unabsorbed serum	W*	CM	C	C	++++	++++	+++	+++	+++	++	+	−
	SH	CG	C	++++	+++	++	+	±	−	−	−	−
	H	CG	++++	+++	++	+	±	±				
Absorbed with shaken suspension	W	++++F	++++	+++	+++	+++	+++	+++	++	+	−	−
	SH	−	−	−	−	−	−	−				
	H	−	−	−	−	−	−	−				
Absorbed with heated suspension	W	+++F	+++	+++	+++	+++	+++	+++	++	+	−	−
	SH	−	−	−	−	−	−	−				
	H	−	−	−	−	−	−	−				
3rd washing of shaken suspension	W	±F	−	−	−	−	−	−				
	H	−	−	−	−	−	−	−				
1st extraction of shaken suspension	W	+++F	+++	+++	++	++	+	−				
	H	−	−	−	−	−	−	−				
3rd washing of heated suspension	W	+F	±	−	−	−	−	−				
	H	−	−	−	−	−	−	−				
1st extraction of heated suspension	W	+++F	+++	++	++	++	+	−				
	H	−	−	−	−	−	−	−				

* M = mixed agglutination; G = granular agglutination; F = floccular agglutination; W = whole antigen; SH = shaken antigen; H = heated antigen.

After absorption with the deflagellated suspension the flocculating agglutinin titer of "whole" antiserum was decreased by one dilution. In other words the shaken and heated suspensions had removed some flocculating agglutinin in spite of the fact that flagellar antigen was entirely lacking. The third washing fluids gave a slight floccular reaction with the whole antigen in low dilution. Both of the extraction fluids agglutinated the whole suspension with a typical floccular clumping through a dilution of 1:64 (1:1,600). There was no reaction with the heated antigen. The extraction of shaken and heated suspensions of *B. paratyphi* Type I sensitized by exposure to homologous serum gave practically the same results. The titer of flocculating agglutinin was reduced following absorption and a flocculating agglutinin was removed upon extraction. The extraction tests apparently confirm the earlier observation that deflagellated suspensions of motile bacteria may absorb some flocculating agglutinin from homologous "whole" antiserum.

The fragility of bacterial flagella suggested a possible explanation, other than that of extraction, for the removal of flocculating agglutinin from the sensitized bacteria. The flagella are particularly sensitive to heat, as demonstrated by Beyer and Reagh (6), Orcutt (7) and others. At high temperature the flagella lose their morphological identity and disintegrate. Heated flagellar suspensions while they no longer give a floccular agglutination with antiserum do give a precipitate in low dilutions indicating the presence of soluble material. Although deflagellated motile bacteria may remove some flocculating agglutinin from "whole" antiserum, the bulk of that agglutinin upon absorption by whole bacteria is taken up by the flagella. The removal of flocculating agglutinin from the sensitized bacteria by the method employed might result from a breaking up of the flagella with subsequent release of the previously combined antibody. A number of experiments were performed to demonstrate the possible relationship of flagellum destruction to agglutinin removal.

The effect of temperatures on either side of that employed in the previous extractions and of high salt concentration on the agglutinability of Type II *B. paratyphi* was first determined.

Heavy suspensions, about twice normal opacity, were prepared in normal saline and in 5 per cent sodium chloride solution. These were heated to 55°,

60° and 65°C. respectively for 1 hour. The heated suspensions were then diluted to normal opacity with saline and tested with "whole" antiserum. The results of the agglutination tests are given in Table IV.

There was no indication that the flagella were affected by the high salt concentration or by a temperature of 55°C. The agglutinability of the organism both in saline and in 5 per cent sodium chloride solution was practically the same as that of a normal unheated sus-

TABLE IV.

Effect of Heat and High Salt Concentration on the Agglutinability of Type II B. paratyphi.

Suspension	Serum dilutions								
	200	400	800	1,600	3,200	6,400	12,800	25,600	51,200
Unheated in saline	CM*	C	++++	++++	+++	+++	++	+	−
Heated 55°C. in saline	CM	C	++++	+++	+++	++	++	+	−
Heated 55°C. in 5 per cent NaCl	CM	C	++++	+++	+++	++	++	+	−
Heated 60°C. in saline	++++M	+++	++	++	+	+	+	±	−
Heated 60°C. in 5 per cent NaCl	++++M	+++	++	++	+	+	+	±	−
Heated 65°C. in saline	++++G	+++	++	+	±	−	−	−	−
Heated 65°C. in 5 per cent NaCl	++++G	+++	++	+	±	−	−	−	−

* M = mixed type of clumping; G = granular type of clumping.

pension. At 60° and 65°C. the destruction of flagella was plainly indicated. At 60°C. both saline and 5 per cent salt suspensions agglutinated to the titer limit of the serum but the intensity of the reaction was much reduced in all dilutions. At 65°C. the type of clumping changed from a predominantly floccular one to a purely granular one. The limiting dilution was much reduced corresponding to that of deflagellated bacteria by "whole" antiserum, as pre-

viously determined. At 65°C. complete destruction of the flagella was indicated.

If the removal of antibody were due to flagellum destruction through the agency of heat then increasing the temperature to 65°C. should result in a greater yield of flocculating agglutinin. On the other hand, reducing the temperature to 55°C. should practically eliminate the removal. A number of extractions were carried out at

TABLE V.

Effect of Varying Temperature and Salt Concentration on the Removal of Agglutinin from Sensitized Type II B. paratyphi.

Method of extraction	Type of antigen	Limit of agglutination	Form of clumping
55°C. in 5 per cent NaCl	W*	1:128	Floccular
	H	<1:2	
55°C. in saline	W	1:64	Floccular
	H	1:2	Granular
60°C. in 5 per cent NaCl	W	1:526	Floccular
	H	<1:2	
60°C. in saline	W	1:256	Floccular
	H	1:2	
65°C. in 5 per cent NaCl	W	1:526	Floccular
	H	<1:2	
65°C. in saline	W	1:256	Floccular
	H	<1:2	

* W = whole antigen; H = heated antigen.

various temperatures and salt concentrations of the diluent. The agglutinin titer of the several extracts is given in Table V. The results are expressed in terms of the highest dilution showing any macroscopic evidence of agglutination.

Heating the sensitized bacteria to 55°C. resulted in the removal of considerable flocculating agglutinin. As noted in Table IV there was no destruction of flagella at that temperature. The yield of free agglutinin was somewhat greater in the presence of 5 per cent sodium

chloride than in saline. With the latter diluent a small amount of granular agglutinin was removed. At 60°C. the yield of floccular agglutinin was increased with both diluents but there was no further increase at 65°C. The complete destruction of flagella at that temperature was previously noted. From the reported observations the removal of flocculating agglutinin was obviously not dependent solely on the destruction of flagella either through the agency of heat or high salt concentration.

The substitution of distilled water for salt solution in the method of extraction likewise resulted in some liberation of agglutinin. The reaction presented a number of peculiarities concerning the mode of action of the extracted antibody. Experiments bearing on the reaction are being continued.

DISCUSSION.

The preceding experiments indicate that the two agglutinins present in "motile" antiserum display certain differences with respect to their removal from sensitized antigen. It was found that the flocculating or flagellar agglutinin could be freed from its combination with antigen over a considerable range of temperature and salt concentration. Suspension of the sensitized bacteria in a 5 per cent solution of sodium chloride followed by heating to 60°C. for 1 hour was particularly favorable for the removal of flocculating agglutinin. The method of titration was not strictly quantitative but the removal of approximately 50 per cent of the combined agglutinin was indicated. With similar treatment there was little or no removal of granulating agglutinin either from sensitized motile or non-motile bacteria.

It may be said that the combining or clumping properties of the granulating agglutinin of "motile" antiserum are not affected by the conditions imposed upon it during extraction. "Motile" antiserum diluted with 5 per cent sodium chloride solution and heated to 60°C. for 1 hour was found to agglutinate heated homologous antigen in as high dilution as did the untreated serum. The content of granulating agglutinin was not noticeably reduced.

Huntoon and Etris (4) had previously claimed that the presence of salt interfered with the removal of agglutinin from sensitized antigens. Their work was confined to the pneumococcus, the menin-

gococcus and *B. dysenteriæ* Flexner. All of these organisms are non-motile and as antigens give rise to granulating agglutinin but not to flocculating agglutinin. With the foregoing experiments the presence of sodium chloride was found to inhibit the removal of granulating agglutinin from sensitized motile and non-motile bacteria. Increasing the concentration of sodium chloride, on the other hand, increased the amount of flocculating agglutinin removed from the sensitized motile bacteria. A 5 per cent solution of sodium chloride gave a greater yield than did an 0.85 per cent solution. The present findings confirm the work of Huntoon and Etris on the inhibitory effect of salt on the removal of that agglutinin which reacted, in their case with non-motile bacteria, and in the present case with non-motile and deflagellated motile bacteria. Their original statement must be modified, however, to include only granulating agglutinin, since the presence of salt favors the removal of flocculating agglutinin.

It was shown that destruction of flagella through the agency of heat with a subsequent liberation of combined agglutinin could not alone explain the dissociative reaction. It was also shown that flagella were not destroyed by the high concentration of salt. At a temperature of 55°C., which does not affect the flagella, a considerable amount of flocculating agglutinin was removed. At 65°C., which results in complete destruction of the flagella, there was no increase over the amount removed at 60°C. The latter temperature is about the critical point for the beginning destruction of flagella by heat.

The method employed was originally chosen as one favorable for the extraction of globulin. It seems probable that the liberation of flocculating agglutinin attendant upon its application is associated with that protein. Experiments bearing on the chemical nature of the extracted antibody were not attempted. While it was free in the sense that it could again unite with specific antigen it still may have retained some cellular fraction from its previous union. Locke and Hirsch (8) commenting on extraction procedures in general state that in most instances no notable dissociation of the antigen-immune substance union is produced but rather a disintegration and dispersion of the sediment, *i.e.* the sensitized antigen.

Failure to remove granulating agglutinin under the conditions of extraction might be due to differences in the chemical nature of the

two agglutinins or to differences in the manner of linkage to their respective antigens.

SUMMARY.

It was shown that flocculating (flagellar) agglutinin and granulating (somatic) agglutinin display certain differences with respect to their removal from sensitized bacteria (*B. paratyphi*). A 5 per cent solution of NaCl added to sedimented, sensitized bacteria followed by heating to 60°C. for 1 hour removed approximately 50 per cent of the combined agglutinin. There was little or no removal of granulating agglutinin either from the sensitized motile bacteria or from a sensitized non-motile organism (*Staphylococcus*). Evidence was presented that the agglutinin removal was not dependent solely on disintegration of flagella by the conditions of extraction with a subsequent freeing of antibody.

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THE ACID-BASE COMPOSITION OF GASTRIC SECRETIONS.*

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Excepting as concerns hydrochloric acid, very few measurements of inorganic factors in the composition of gastric secretions have been published. Gamble and Ross (1) found sodium in vomited stomach secretions from a dog following experimental obstruction of the pylorus to the extent of quite one-half the equivalence of the chloride ion present. In a study of the effects of pyloric obstruction in rabbits, Gamble and McIver (2) showed that more than twice the entire plasma content of fixed base was lost into the stomach during the survival period. They regard this extensive withdrawal of fixed base as the cause of the accompanying rapid dehydration of the blood and interstitial body fluids. The data to be here presented were obtained with the purpose of learning whether or not fixed base is contained in stomach secretions, produced under approximately normal conditions, to an extent which would explain a large loss of base in the presence of circumstances preventing reabsorption of these secretions.

So far as we are aware, the only satisfactorily complete analyses of gastric juice are those of Rosemann (3). The material studied was "hunger juice" obtained from dogs by the sham feeding method of Pawlow. An appreciable amount of fixed base was found. The secretion of an alkaline juice by the mucosa of the pyloric antrum was noted by early workers and has recently been confirmed by Ivy and Ayama (4), and by Limb and Dott (5). The production of an alkaline mucous secretion is of course not confined to the pyloric region. Beaumont (6) noted that the inner coat of the stomach of his amiable *voyageur* was "constantly covered with a very thin, transparent, viscid mucus, lining the whole interior of the organ," and that "on applying the tongue to the mucous coat of the stomach, in its empty, unirritated state, no acid taste can be perceived."

* A partial report of the findings presented in this paper was published in the *Proceedings of the Society for Experimental Biology and Medicine*, 1926, xxiii, 439, under the title, "Fixed base in gastric juice."

The animals used in our experiments were cats. The secretions analyzed were obtained from isolated pouches constructed in the fundic and pyloric regions of the stomach. The animals were operated under ether anesthesia with aseptic technic. During the recovery period, they were cared for in warm, well bedded cages. The pouches were provided with external fistulæ and were found to retain secretions entering them until removed by catheterization. The greater part of the material studied was obtained from an animal in which a pouch of the Heidenhain type had been established in the fundus of the stomach. Samples of juice were taken from this pouch over a period of 2 months, the animal remaining in an excellent state of nutrition and apparently suffering no discomfort.

Chemical Methods Used.

Total fixed base, Fiske (8); *chlorides*, Fiske (9); *inorganic phosphate*, Fiske and Subbarow (10); *potassium and calcium*, Tisdall and Kramer (11). *Sodium* was taken as total fixed base—(potassium plus calcium). The magnesium factor in total fixed base, which according to the measurements of Rosemann (3) is relatively minute, was disregarded. *Nitrogen* was determined by the micro Kjeldahl method of Folin and Denis (12).

The secretions obtained from the pouch during active digestion of food in the stomach were colorless and water-clear. It was found that if the pouch was well drained of these secretions no material could be obtained from it by subsequent catheterization after intervals of fasting. Evidently the secretion of the resting mucosa did not accumulate sufficiently to permit obtaining a sample for analysis. The existence of this secretion could however be made clearly evident by allowing juice produced after ingestion of food to remain in the pouch past the period of active digestion in the stomach. This juice, on being withdrawn, instead of being water-clear always showed a faint but definite white cloudiness, and on analysis a relatively extensive gain in organic material and in fixed base was found. In Table I are given the results of analyses of three large specimens of secretions taken from the pouch at differing intervals after ingestion of food. Each specimen was composed of a number of separate collections of juice. The table also contains Rosemann's data from pooled collections of "hunger juice" from a dog. As may be seen in

the table, the inorganic factors in the juice collected during active digestion of food in the stomach (Specimen 1) correspond closely with the values found by Rosemann for "hunger juice" in the dog. In the specimens composed of juice allowed to remain in the pouch after completion of active gastric function (Specimens 2 and 3) relatively much larger amounts of organic material and of fixed base were measured. This finding together with the change in the physical

TABLE I.

Data from Secretions Taken from Isolated Pouch in Gastric Fundus of Cat and Rosemann's Data for "Hunger Juice" from a Dog.

	Specimen 1 2–4 hrs. after food. Water-clear	Specimen 2 8–12 hrs. after food. Faint milkiness	Specimen 3 18 hrs. after food. Moderate milkiness	Hunger juice from dog. Rosemann
	gm. per 100 cc.	*gm. per 100 cc.*	*gm. per 100 cc.*	*gm. per 100 cc.*
Dried substance	0.169	0.282	0.649	0.387
Ash	0.121	0.179	0.384	0.131
Organic substance	0.048	0.103	0.265	0.256
Nitrogen	0.010	0.018	0.041	
Protein (N × 6.25)	0.063	0.113	0.256	
Inorganic substances: Cl	0.588	0.574	0.552	0.630
P	0.00016	0.00019	0.00055	0.00027
Na	0.028	0.045	0.128	0.022
K	0.045	0.053	0.053	0.037
Ca	0.0106	0.0057	0.0041	0.00015
Total	0.672	0.678	0.781	0.689
Organic + inorganic substances	0.720	0.781	1.003	0.945

appearance of the specimens from water-clear to "faint" and "moderate" milkiness would seem definitely to indicate admixture of the mucous secretion of the resting mucosa with the acid juice from the fundic glands. The increase of fixed base, it may be noted, is composed of sodium, the values for potassium remaining approximately stationary.

In Table II are given measurements of chloride ion, of fixed base and in a few instances of potassium obtained from single collections of juice from the pouch. The first two groups of measurements are

TABLE II.

Data from Secretions Taken from Fundic Pouch of the Heidenhain Type.

Specimen No.	Food	Interval after food	Volume	Cl′	B·	H·	K·
		hrs.	*cc.*	*cc. 0.1 N per 100 cc.*	*cc. 0.1 N per 100 cc.*	*cc. 0.1 N per 100 cc.*	*cc. 0.1 N per 100 cc.*
1	Meat	1	2	160	22	138	
2	"	2	7	166	17	149	
3	"	3	4	167	24	143	
4	"	6	8	168	44	124	
5	Fish	1	17	177	49	128	
6	"	2	9	156	31	125	12.5
7	"	2	14	169	75	94	
8	"	4	3	157	39	118	
9	"	2	9	171	32	139	
10	"	2	11	169	30	139	11.7
11	"	2	21	173	24	149	11.8
12	"	4	3	152	35	117	11.6
13	Milk	4	28	174	46	128	
14	Bread and milk	2	10	156	41	115	
15	" " "	3	18	176	25	151	
Average.....		**3.7**	**11**	**166**	**36**	**140**	**12**
16	Cream	1	3	161	49	112	
17	"	2	2	160	42	118	
18	"	3	2	162	38	124	
19	Cereal and cream	6	10	160	72	88	14.6
20	" " "	6	14	162	77	85	10.4
21	" " "	8	8	157	83	74	9.6
22	" " "	6	7	151	80	70	8.9
23	" " "	2	8	167	71	96	10.9
24	Bread and water	2	4	165	65	100	
25	" " "	4	3	186	55	131	
26	" " "	2	6	162	48	114	
Average.....		**3.8**	**6**	**163**	**62**	**101**	**11**
27		18	45	160	53	107	
28	Morning	18	24	155	72	83	12.1
29	specimens	18	13	158	89	69	
30		18	8	157	139	18	15.6
31		18	5	164	142	22	11.8
Average.....		**18**	**19**	**159**	**99**	**60**	**13**

from samples of juice taken from the pouch within 6 hours after ingestion of food, with the single exception that Specimen 21, was collected 8 hours after feeding. The food given the animal before collecting the samples in the first group was meat, fish or milk. These were the foods given while collecting the pooled material from which the measurements in Table I were obtained. The second group of data in Table II are from samples of juice produced following ingestion of food low in protein and without appreciable amounts of buffer salts. From the measurements of phosphate and the estimations of protein given in Table I it is evident that these anions are, as compared with chloride, present in negligible amounts. The concentration found for chloride ion minus that for fixed base will therefore closely describe the concentration of hydrogen ions. This datum, taken as $Cl'\text{-}B^{\cdot}$, is also given in the table. The third group of measurements in Table II are from samples of juice taken from the pouch in the morning before feeding, different amounts of food having been given the evening before.

The outstanding finding from these data is a wide range of fixed base concentration in the presence of a fairly stationary concentration for chloride ion. It is thus evident that variation in acidity of the juice as measured by concentration of hydrogen ions is referable to change in fixed base. The data in the first and second sections of the table are from samples taken from the pouch during active digestion of food in the stomach. The considerable difference in the average values for fixed base for the two groups suggests an adjustment of this factor according to the character of the ingested food. The juice produced during digestion of acid-binding foods contains appropriately less of fixed base than is found when foods low in protein and buffer salts are given. It must be noted however that the individual measurements are in both groups often wide of the average value. If the fixed base in the juice is wholly or partly derived from the mucous secretion of the fundus, the collection interval used and the volume of the specimen obtained are probably factors in the extent to which fixed base is added to the juice from the fundic glands. As may be seen in the table, the intervals allowed for secretion of the specimen following ingestion of food were not uniform and also there was an unaccountable variation in the amounts of juice produced. No

regularity of relationship between these factors and the concentrations of fixed base found, can be made out from these data. By prolonging the collection period past the period of active digestion there occurs, as has already been noted, an increase of fixed base concentration. The data from the "morning specimens" given in the third section of Table II will serve to indicate that the increase of fixed base is fairly closely proportional to the size of the specimen and this finding may be taken as additional evidence that the fixed base found is, in great part at least, derived from the mucous secretion of the fundus. In Table III are given data from specimens of juice collected from the pouch during the digestion of food to which had been added a relatively

TABLE III.

Data from Secretions taken from Fundic Pouch during Digestion of Food Containing Added Sodium Bicarbonate.

Specimen No.	Food	Interval after food	Volume	Cl′	B·	H·
		hrs.	*cc.*	*cc. 0.1 N per 100 cc.*	*cc. 0.1 N per 100 cc.*	*cc. 0.1 N per 100 cc.*
32		1	4	166	60	106
33	Fish, 30 gm.,	4	8	166	73	93
34	+	6	6	176	50	126
35	$NaHCO_3$, 2 gm.	2	7	173	53	120
36		4	2	158	43	115
Average.....		3.6	5	168	56	112

large amount of sodium bicarbonate. As may be seen, there was no compensating increase in the acidity of juice.[1] The fixed base concentrations found are actually somewhat higher than when the same food without added alkali was given (first section of Table II). It is thus evident that we cannot regard the data given in the first and second sections of Table II as dependably indicating that gastric juice acidity is regulated with reference to the character of the food intake by adjustment of fixed base. The differing amounts of fixed base found are probably due to admixture in varying degree of the

[1] This finding is perhaps of some clinical significance with regard to bicarbonate therapy in gastric hyperacidity in that it may be taken to indicate that sodium bicarbonate is not an aggravating stimulus.

mucous secretion with "true" juice. An accurate control of gastric acidity by a regulated mixing of these two secretions is a conception rather difficult to accept.

The results of an experiment given in Table IV indicate that the production of the mucous secretion of the fundus may be greatly increased by the presence of an injurious agent and suggest that an important rôle of this secretion is protection of the mucosa against

TABLE IV.

Data from Secretions Taken from Fundic Pouch Following Irritation by Alcohol.

Interval after irritation by alcohol	Volume of specimen	Cl′	B·	H·
	cc.	*cc. 0.1 N per 100 cc.*	*cc. 0.1 N per 100 cc.*	*cc. 0.1 N per 100 cc.*
15 min.	2	124	119	5
1 hr.	4	135	99	36
2 hrs.	2	141	90	51

TABLE V.

Data from Secretions Taken from Fundic Pouch Constructed According to the Technic of Pawlow.

Specimen No·	Cl′	B·	H·
	cc. 0.1 N per 100 cc.	*cc. 0.1 N per 100 cc.*	*cc. 0.1 N per 100 cc.*
37	170	36	134
38	170	30	140
39	175	32	143
40	166	32	134
Average.....	170	32	138

harmful irritation. The experiment consisted in introducing into an empty fundic pouch a small quantity of 95 per cent alcohol which was then almost immediately withdrawn.[2] This procedure induced secretions to an extent sufficient to provide several successive samples large enough for analysis. As may be seen in the table, the chloride

[2] This experiment was carried out with the animal from which the data in Table II were obtained.

ion concentration was greatly below the usual value and was almost covered by fixed base. Over an interval of 2 hours there occurred a considerable increase of chloride and a decrease of fixed base. These data suggest that measurements of fixed base as well as of chloride ion might be informative in the study of gastric anacidity.

In Table V are given data from several specimens taken from a pouch constructed according to the technic of Pawlow, by which vagus innervation of the pouch is preserved. The food given this animal was fish and the specimens were collected 4 hours after ingestion. As may be seen in the table the values found for chloride and fixed base agree fairly closely with those obtained from juice taken from the Heidenhain type of pouch.

In a third animal, a pouch was constructed in the region of the pyloric antrum and from it small amounts of a colorless and viscid secretion were obtained by prolonged catheterization. From a specimen composed of a number of separate collections, the following measurements per 100 cc. were obtained: $B^{\cdot}$, 169 cc. 0.1 N, Cl', 158 cc. 0.1 N, $K^{\cdot}$, 8.8 cc. 0.1 N, dried substance, 2.49 gm. The reaction of the material as determined colorimetrically was pH 8.4. Doubtless, however, this degree of alkalinity was in part due to loss of CO_2. This secretion is thus seen to contain fixed base at approximately the concentration found for chloride ion in juice from the fundus of the stomach, and to owe its alkalinity to a slight recession of chloride ion from this level. The extent to which fixed base is in excess of chloride ion, 11 cc. 0.1 N, may probably be taken as an approximate measurement of bicarbonate ion. In Table II, as in Table I, it may be noted that the values for potassium being approximately stationary, variation in the amounts of fixed base is evidently referable to sodium. The specimen from the pyloric pouch contains potassium at about the level found in the secretions from the fundus. The probability that the mucous secretion of the resting mucosa of the fundus has the same composition as that of the pyloric antrum is thus indicated.

The above data describing the acid-base composition of the gastric secretions are graphically summarized in Fig. 1. In the diagrams, the anions compose the right-hand and the cations the left-hand column. The figure also contains a diagram of the acid-base composition of

cat's blood serum.[3] The first three diagrams illustrate clearly the range of hydrogen ion concentration in secretions from the fundic pouch produced by change in fixed base in the presence of an approximately stationary concentration of chloride ion. It is perhaps interesting to note that whereas in the blood plasma the total ionic content is determined by the fixed base concentration owing to the

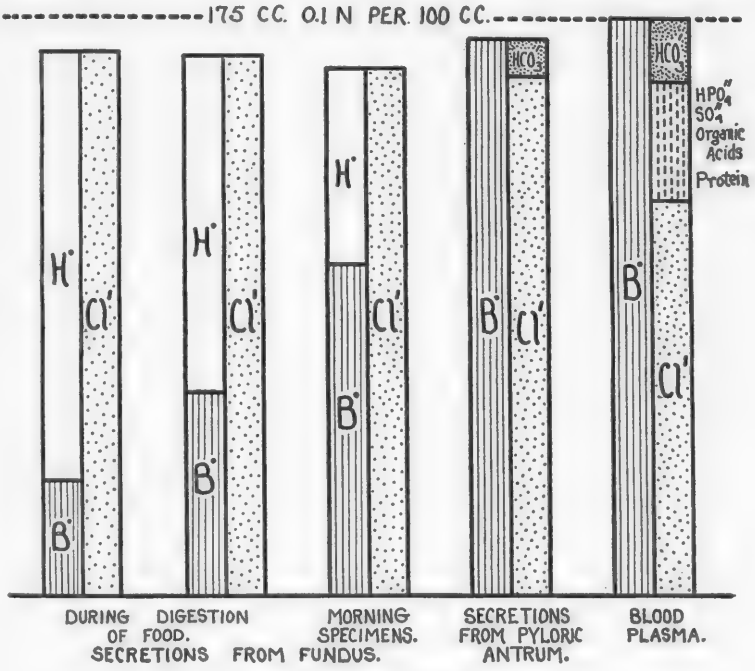

FIG. 1. From data in Table II.

adjustability of the acid factor (HCO_3'), in the secretions of the gastric fundus this value is established by chloride ion and the movable factor sustaining acid-base equivalence (H') is on the base side of the diagram. An approximate isotonicity of gastric juice and blood

[3] Except for the bicarbonate value which is the average of a few measurements made in this laboratory, the data published by Baumann and Kurland (7) were used in constructing this diagram.

serum has been indicated by measurements of freezing point depression. By comparing the heights of the diagrams in the figure it is evident that according to these data the total ionic content of cat's blood serum is appreciably greater than was found for the gastric secretions. A Donnan relationship between physiological solutions separated by secreting cells would not be expected. The fact, however, that the total of ions is here greater in the solution containing protein and that the chief anion chloride is found at higher concentration in the protein-free solution might be taken as a vague hint of Donnan control of at least the factors defining total ionic content.

A glance at the composition of the gastric secretions as displayed in these diagrams will make easily understandable the large withdrawal of fixed base as well as of chloride ion from the blood plasma which may occur when reabsorption of these materials is prevented. Irritant circumstances in a fasting animal, experimental obstruction of the pylorus for instance, may be expected to produce a relatively large amount of the alkaline mucous secretion. It is therefore not surprising that, as has been found, a chloride loss may be accompanied by more than half its equivalence of fixed base.

SUMMARY.

The chief inorganic factors in secretions obtained from isolated pouches constructed in the fundus and in the pyloric antrum of the cat's stomach were found to be chloride ion and fixed base. In a series of samples obtained from the fundic pouch, chloride ion was approximately stationary at 165 cc. 0.1 N per 100 cc. During digestion of food in the stomach, secretions from the pouch contained fixed base in amounts varying considerably from an average of 47 cc. 0.1 N per 100 cc. Material allowed to remain in the pouch after the completion of food digestion in the stomach showed an increasing content of fixed base, to as much as 140 cc. 0.1 N per 100 cc. A stationary total ionic content of secretions of the fundus is thus seen to be sustained by the chloride ion concentration, and changes in hydrogen ion concentration to be caused by variation of fixed base. The differing amounts of fixed base found are regarded as probably due to admixture of a mucous secretion with the juice from the fundic glands. The alkaline secretion taken from a pyloric pouch contained

fixed base in excess of chloride ion. Variation of fixed base in the secretions from the fundic pouch were found to be referable to change in sodium content, the smaller factor, potassium, remaining approximately constant at about the value found in material from the pyloric pouch. This suggests that the mucous secretion of the fundus has the same composition as that produced by the pyloric antrum. These data will serve to explain the extensive withdrawal of fixed base, as well as of chloride ion, from the blood plasma in the presence of circumstances causing a continued loss of stomach secretions.

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ACID-BASE COMPOSITION OF PANCREATIC JUICE AND BILE.

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The measurements of fixed base in gastric secretions given in the preceding paper, explain a rapid depletion of blood plasma base when these secretions are continuously lost from the stomach. It has been pointed out that this loss of fixed base, rather than the larger loss of chloride ion, is the significant factor in the accompanying dehydration of the plasma (1). The alkaline digestive juices entering the duodenum presumably contain more of fixed base than of chloride ion. Under normal circumstances the inorganic substances contained in these secretions are in chief part returned to the blood plasma. If the concentrations of these substances in the digestive juices are appreciable in terms of their blood plasma values, continued failure of reabsorption due to abnormal circumstances such as upper intestinal obstruction, fistula, or diarrheal disease, may be expected to produce dehydration of the plasma and, also, distortion of acid-base structure, unless the amounts of the individual substances lost are proportional to their plasma concentrations. In this study it was undertaken to establish the concentrations of the chief plasma substances, fixed base and chloride ion, in pancreatic juice, in bile from the hepatic duct and from the gall bladder, and in secretions accumulating in an upper intestinal loop. The materials studied were obtained from dogs and from cats by the operative procedures described below. Two specimens of human pancreatic juice were obtained from a patient with a pancreatic fistula.

Collection Methods.

Pancreatic Juice.—An unusual surgical situation provided the opportunity of collecting two samples of human pancreatic juice. This material was obtained

from a patient at the Massachusetts General Hospital, who had undergone an operation for removal of the contents of a large pancreatic cyst. The walls of the cyst were stitched to an opening in the anterior abdominal wall and for a time a sinus tract discharging a clear mucoid fluid persisted. In addition to this material, a clear, watery fluid was noted to leave the sinus after meals. Two small collections of this latter material were obtained following breakfasts of milk, cream, tea, and toast. Tests for the specific ferments of pancreatic juice in the fluid were positive.

A few samples of juice were obtained from the cat, but this animal was found to be unsatisfactory both as regards the establishment of a fistula and the subsequent withdrawal of juice. In the formation of the fistula, it is necessary to use the main pancreatic duct. Since this duct opens into the duodenum at the papilla of Vater close to the bile duct, it is necessary to side-track the bile in order to obtain the pancreatic juice alone. Furthermore, the lumen of the intestine is so small that it is not possible to excise the portion containing the pancreatic duct and then close the wall, so that the intestinal stream, as well as the bile, must be diverted from the first portion of the duodenum. This was done in stages as follows: Under ether anesthesia, an anastomosis was made between the gall bladder and the stomach, and a posterior gastroenterostomy between the stomach and the jejunum was carried out. 4 weeks later, the animal having completely recovered from the operation, the common duct was divided, the bile now passing from the gall bladder into the stomach. The portion of the duodenum containing the pancreatic duct was then excised for transplantation into the abdominal wall as a permanent fistula. The distal end of the duodenum was closed by suture. The stomach contents then entered the intestine through the posterior gastroenterostomy. Except for loss of weight, the animals remained for 2 weeks in fair condition. There appeared to be a continuous slight secretion of pancreatic juice from the fistula which greatly increased on taking food.

The dog with us, as with other investigators, proved in all respects satisfactory. The fistula was established by two methods. The first, that of Pawlow (2), is as follows: A small section of the duodenal wall containing the accessory pancreatic duct is excised; the opening in the duodenum is closed, and the section of the duodenum containing the mouth of the duct transplanted into the abdominal wall.

The second method is an adaptation by Elman and McCaughan (3) of the Rous and McMaster method (4) for collecting sterile bile, and is practically identical with the latter, except that the pancreatic duct is used. Briefly, it consists in cannulation of the pancreatic duct and collection of the secretion in a balloon outside the abdomen. The connection between the cannula and the balloon is made by a somewhat elaborate series of rubber tubes which insures collection of the juice under aseptic conditions. It has the disadvantage, pointed out by Pawlow (2), in regard to methods involving cannulation of the duct, that the cannula acts as a constant irritant, perhaps stimulating abnormal secretion.

Bile.—The bile studied was obtained from cats, collections being made from both the gall bladder and the hepatic duct. The gall bladder collections were made as follows: After a 24 hour fast, the animal was etherized, the abdomen opened and the cystic duct occluded by a clamp. A needle attached to a syringe was then introduced into the bladder and the contents aspirated. The hepatic duct collections were made by an adaptation of the method of Rous and McMaster (4). The gall bladder was first removed and the cystic duct ligated. The hepatic duct was divided and a cannula placed in the proximal end, the end towards the duodenum being ligated. The cannula was connected, by a system of rubber tubing, with a balloon outside the abdomen, an arrangement permitting collection of the entire biliary secretion.

Upper Intestinal Secretions.—A loop about 20 cm. from the pylorus was selected and the bowel divided. A second cut was made through the bowel about 25 cm. distal to the first one, leaving the loop attached by the mesentery only. Great care was used not to interfere with the blood supply. The continuity of the gastrointestinal tract was then restored by an end to end anastomosis and the ends of the isolated loop closed. A small opening into the lumen of the loop was made, and a catheter inserted and held in place by purse string sutures, the free end being brought out through a stab wound in the abdominal wall and connecting with a balloon.

All operations were carried out under ether anesthesia and with aseptic technic. The animals were then cared for in comfortable cages and at the end of the experimental period were killed by etherization.

Methods of Analysis.

The methods used for measuring total fixed base, chloride, inorganic phosphate and calcium, were those cited in the preceding paper. The measurements of sodium and potassium given in Table II were obtained by the method of Stoddard (5) for determining sodium plus potassium and the method of Fiske (6) for potassium.

RESULTS.

The measurements of the two chief factors, fixed base and chloride ion, in the acid-base composition of the secretions studied are given in Table I. The diagrams in Fig. 1 were constructed from average values from these data. The figure also contains for comparison, diagrams representing the acid-base composition of gastric juice and of blood plasma. In the specimens of pancreatic juice, the concentration of fixed base was found to be double or more that of chloride ion. The single measurement of fixed base in human pancreatic juice gave a value only slightly above that usually found for

fixed base in human blood plasma (158 cc. 0.1 N per 100 cc.). The averages for fixed base in the series of samples from Dog 3 and from Dog 4, showed a rather wide difference, being respectively 182 cc. 0.1 N and 159 cc. 0.1 N per 100 cc. Measurements of fixed base in the blood plasma of these animals, however, gave approximately corresponding levels, the values found being for Dog 3, 175 cc. 0.1 N per 100 cc., and for Dog 4, 159 cc. 0.1 N per 100 cc. This degree of difference in the blood plasma level of fixed base in these two animals is surprising and, we believe, unusual. The fixed base found in

TABLE I.

Measurements of Fixed Base and of Chloride Ion.

Material	Number of specimens analyzed	B', cc. 0.1 N per 100 cc.			Cl', cc. 0.1 N per 100 cc.		
		Min.	Max.	Av.	Min.	Max.	Av.
Pancreatic juice, human	2	—	—	164	86	88	87
" " dog, No. 3	19	170	189	182	28	66	42
" " " " 4	6	152	163	159	55	89	73
" " cats (two)	2	192	200	196	67	93	80
Bile, hepatic duct, cats	11	157	194	172	100	130	120
" gall bladder "	8	261	318	274	0	20	5
Upper jejunal loop, cat*	2	169	172	170	129	131	130
Blood plasma, cat**		—		175	—	—	119

* Inflammation due to infection was a factor in production of fluid taken from the jejunal loop.

** From measurements published by Baumann and Kurland (7).

single samples from each of the two cats, 192 cc. 0.1 N and 200 cc. 0.1 N per 100 cc., is considerably above the usual plasma concentration in this animal, 175 cc. 0.1 N per 100 cc., established by the data of Baumann and Kurland (7). The specimens of pancreatic juice, however, were small, and the measurements obtained from them cannot be taken as dependably defining the usual concentration of fixed base in pancreatic juice from the cat.

Detail of the acid-base composition of a sample of pancreatic juice from Dog 4 is given in Table II. As may be seen, the fixed base is

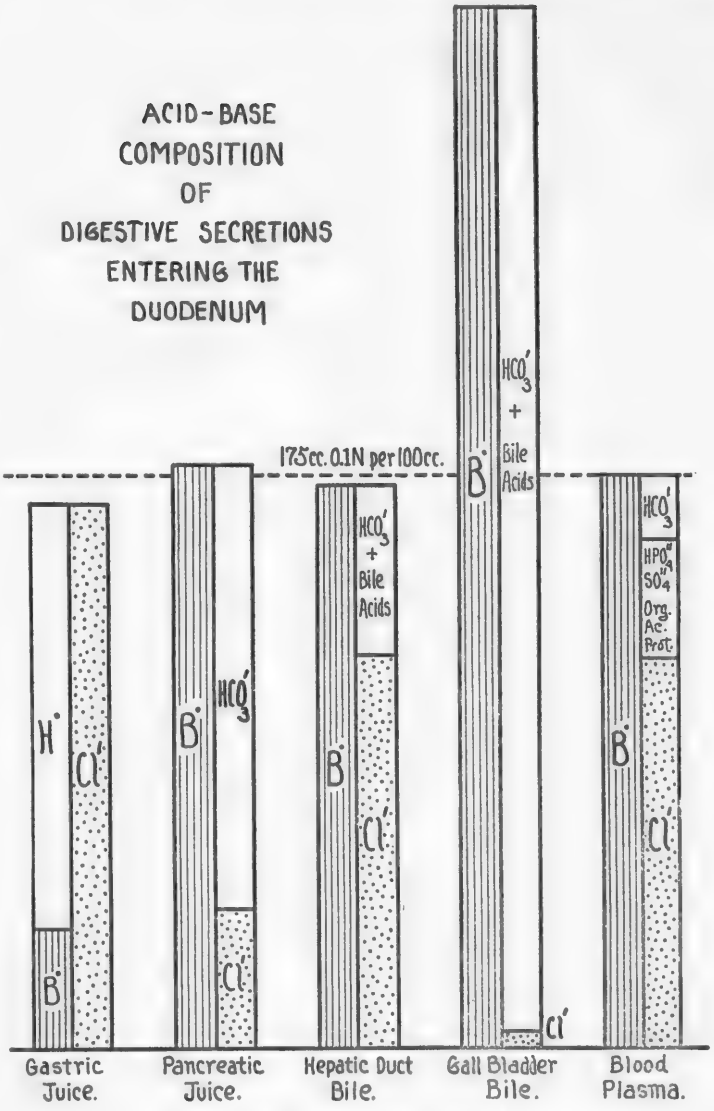

FIG. 1. From data in Table I.

composed almost entirely of sodium, potassium and calcium being found at relatively small values. Magnesium was not measured. Except for a minute concentration of phosphate, the acid factors are chloride ion and bicarbonate ion. In this table the concentration of HCO_3' is assumed to be measured by the extent to which fixed base is in excess of $Cl' + HPO_4''$. That this surmise is approximately correct is indicated by several measurements of bicarbonate obtained

TABLE II.

Acid-Base Composition of Pancreatic Juice from Dog.

HCO_3' taken as total base—$(Cl' + HPO_4'')$. Magnesium not measured.

Base		Acid	
	cc. 0.1 N per 100 cc.		*cc. 0.1 N per 100 cc.*
$Na^{\cdot}$	148	Cl'	81
$K^{\cdot}$	7	HPO_4''	1
$Ca^{\cdot\cdot}$	6	HCO_3'	79
	161		161

TABLE III.

Measurements of Bicarbonate in Pancreatic Juice after Equilibration with Alveolar Air Compared with Values for Total Fixed Base Minus Chloride.

Material from Dog 4.

Specimen No.	$B^{\cdot} - Cl'$	HCO_3'
	cc. 0.1 N per 100 cc.	*cc. 0.1 N per 100 cc.*
1	96	99
2	88	98
3	81	87
4	63	69

by a somewhat rough method from samples of pancreatic juice in which fixed base and chloride were also determined. The measurements were obtained by first shaking samples of juice in the Van Slyke CO_2 apparatus with alveolar air from the operator and then determining bicarbonate in the usual manner. As may be seen in Table III, the measurements were somewhat beyond the mark but may be taken as satisfactorily demonstrating that the acid equivalence of the base in pancreatic juice is composed practically entirely of chloride and bicarbonate.

The data from the series of specimens of pancreatic juice from Dog 3 and from Dog 4 (Table I) demonstrate that fixed base is, for the individual, a much more stationary value than is chloride ion. Pawlow (2) and others have stated that the alkalinity of pancreatic juice varies considerably, apparently according to differences in the character of the food intake. Fixed base remaining stationary, adjustment of bicarbonate and thereby degree of alkalinity, is referable to change in the chloride ion concentration. The mechanism establishing reaction is thus the inverse of that found in gastric secretions where, as shown in the preceding paper, chloride ion is stationary and fixed base is the movable factor.

In bile from the hepatic duct, fixed base and chloride were found at approximately the concentrations which obtain in the blood plasma (see Fig. 1). The fixed base concentration in bile from the gall bladder exhibits, interestingly, a wide departure from the blood plasma level, reaching in one of the specimens a value of 318 cc. 0.1 N per 100 cc. The work of Rous and McMaster (4), which has shown us that the gall bladder has the unique function of concentrating a physiological secretion, would lead us to expect this finding. Another striking and ingenious change in the composition of gall bladder bile is the almost complete removal of chloride ion, with presumably, replacement by bile acids.[1]

It is the evidence of these data that, during digestive activity in the upper intestinal tract, much more of fixed base than of chloride ion is required. In the case of pancreatic juice, these materials are directly withdrawn from the blood plasma. The release of gall bladder bile with its large content of fixed base does not, however, involve an immediate and extensive removal of plasma base. Replacement of bladder bile is presumably a gradual process extending over the period of absorption of fixed base from the gastrointestinal tract and

[1] In the diagrams representing the composition of the bile specimens the presence of bicarbonate is indicated. No measurements of bicarbonate were made. From the studies of Rous and McMaster of the reaction of bile, we are informed that gall bladder bile is nearly neutral or even slightly acid, and hepatic duct bile, or "liver bile" to use their more appropriate term, is decidedly alkaline. It may therefore be inferred that the former contains a very small amount and the latter a considerable amount of bicarbonate.

is probably accomplished without disturbing the fixed base content of the plasma. We may thus regard the reservoir mechanism for supplying the digestive secretion of the liver as providing a probably important protection against sudden and large depletions of plasma base. It is to be noted however that, in the presence of circumstances causing continued loss of bile, extensive withdrawal of plasma fixed base must occur unless the food intake provides adequate replacement.

The experiment of constructing a jejunal loop with fistula, continuity of the remainder of the gastrointestinal tract being preserved, was undertaken with the purpose of learning the inorganic composition of the secretions of the upper intestinal mucosa during the digestion of food. The experiment was unsuccessful. It was found that moderate infection and inflammation were undoubtedly factors in the production of the fluid which was obtained from the loop in surprisingly large amounts. The measurements obtained are given in the table for the reason that they probably indicate the composition of the fluid which is so abundantly produced by the intestinal mucosa under obstructive conditions. As may be seen both fixed base and chloride ion are present at approximately their respective concentrations in blood plasma.

SUMMARY.

Pancreatic juice contains fixed base at approximately the concentration found in the blood plasma. Chloride ion is present in concentrations varying from one-fourth to one-half the fixed base value and the remainder of the acid equivalence is composed of bicarbonate ion. Fixed base being a nearly stationary factor, variation of bicarbonate and thereby of alkalinity is referable to change in the concentration of chloride ion.

In bile, as delivered by the liver, both the fixed base and chloride ion values correspond closely with their respective concentrations in blood plasma. In gall bladder bile, however, the concentration of fixed base is, roughly, double that in hepatic duct bile, and chloride ion has been almost entirely removed.

From these data it may be inferred that loss of digestive secretions entering the duodenum will, in the absence of replacement of the

materials contained, cause dehydration of the blood plasma and reduction of the plasma bicarbonate.

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BODY FLUID CHANGES DUE TO CONTINUED LOSS OF THE EXTERNAL SECRETION OF THE PANCREAS.

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The body fluid changes caused by continued loss of gastric secretions have been fairly well defined by the experimental work of a number of investigators (1–3). The outstanding alterations are dehydration of the interstitial body fluids and of the blood plasma, and alkalosis. These changes are referable to a continued withdrawal of fixed base and of chloride ion from the blood plasma. The data in the preceding paper demonstrate that the digestive secretions entering the duodenum also contain these plasma substances in appreciable amounts. Presumably loss of these secretions, in the absence of adequate replacement of the materials contained, will cause disturbances of body fluid volume and structure. The data to be presented in this paper describe an experimental attempt to define the body fluid changes developing as a result of continued loss of the external secretion of the pancreas.

The general effects accompanying complete loss of pancreatic juice were first observed by Pawlow (4). He found that dogs with pancreatic fistulæ die unless certain measures are taken. To quote from his description of an experiment: "3 to 4 weeks after the operation, the animal previously well to all appearances, became suddenly ill. Food was almost at once refused and a rapidly increasing disability supervened . . . . followed after 2 or 3 days by death. . . . . There remained but one supposition, *viz.*, that the animals, in the escape of pancreatic juice, lost something essential to the normal processes of life." Pawlow made the significant observation that this train of events could be prevented by placing sodium bicarbonate in the food, and also noted that the survival period was much longer on a diet of bread and milk than when meat alone was given. Elman and McCaughan (5) have recently withdrawn and collected the external secretion of the pancreas by an ingenious method involving cannulation of the

duct. The survival period of their animals (dogs) was very much shorter than observed by Pawlow, probably because of the development of persistent vomiting, an event which, according to Pawlow's experiments and our own, does not occur when the external secretion is withdrawn by means of a fistula.

The findings to be here described were obtained from two experiments with dogs. In the first experiment a Pawlow fistula was constructed using the accessory pancreatic duct. The external secretion lost was therefore only a part of the total production. The diet was bread and milk until the 37th day of the experiment when it was changed to meat. Except for a gradual decline in weight, this animal remained in good condition for 38 days. Symptoms of muscular weakness, apathy, and loss of appetite then developed progressively until the animal was *in extremis* on the 42nd day after operation. In the animal used in the second experiment the main and accessory pancreatic ducts were found united before entering the duodenum so that all of the external secretion was drained from an external fistula of the Pawlow type. The diet given was exclusively meat. The survival period was very much shorter than in Experiment I. The animal was active and apparently in good condition for 10 days and then became progressively weaker and more apathetic, and took little or no food or water. 15 days after operation the animal was in serious condition, showing symptoms of marked prostration; extremities cold, pulse rapid, respiration increased in depth and frequency, and blood pressure so low that it was impossible to obtain a blood sample until the femoral artery had been exposed under novocaine anesthesia. At what seemed to be almost the end of the survival period each of these animals was given large amounts of salt solution by subcutaneous injection and intravenous injections of sodium bicarbonate solution. The surgical part of these experiments was carried out under ether anesthesia, and with aseptic technic. The animals were then placed in comfortable cages and distilled water provided for drinking. When the end of the survival period was evidently near, they were killed by etherization.

Chemical Methods Used.

Plasma bicarbonate was determined by the method of Van Slyke (8) and fixed base by the method of Fiske (9). An unpublished method by Fiske was used

in measuring plasma chlorides. Sodium and potassium in urine and in the horse meat used as food were determined together by the electrolytic method of Stoddard (10) and potassium was separately measured by the method of Fiske (11).

The data obtained from these two experiments are presented in Tables I and II and those from Experiment II which describe the acid-base changes found in the blood plasma are repeated graphically in Fig. 1. As may be seen in the tables the earliest change noted is

TABLE I.

Data from Experiment I.

The value R' represents the remainder of the acid equivalence of total fixed base after subtracting $HCO_3' + Cl'$.

Day after operation	Body weight	Food	Blood plasma			
			B	HCO_3'	Cl'	R'
	kg.		*cc. 0.1 N per 100 cc.*	*cc. 0.1 N per 100 cc.*	*cc. 0.1 N per 100 cc.*	*cc. 0.1 N per 100 cc.*
16	17.6	Bread and milk	175	26	110	39
22	17.8	" " "	175	30	106	39
32	17.2	" " "		24	101	
36	17.2	" " "		24	104	
37	16.8	Meat				
39	16.3	"		21		
41	15.3	"	161	13	105	43
42		"	146		101	
43	15.3	"	139	11	99	29
44*	15.3	Bread and milk				
45**	15.1	" " "	155	21	103	31

* 1000 cc. 0.9 per cent NaCl solution injected into peritoneal cavity. 100 cc. 2 per cent $NaHCO_3$ solution given intravenously.

** 5 gm. $NaHCO_3$ given in milk.

decline of body weight. This begins directly following establishment of the fistula and proceeds gradually until the sudden development of anorexia, when it is greatly accelerated. The data on this point are complete only in Experiment II. The plasma factors studied were found not to be appreciably altered during approximately the first two-thirds of the experimental periods. Then, and corresponding with the sudden appearance of the symptoms described above, extensive change in plasma factors developed rapidly.

Except as regards the concentrations of bicarbonate and of chloride ion, these changes resemble closely those produced by continued loss

TABLE II.

Data from Experiment II.

The value R' represents the remainder of the acid equivalence of total fixed base, B', after subtracting $HCO_3' + Cl'$.

Day after operation	Body weight	Meat intake	Water intake	Blood		Blood plasma						
				Hematocrit	Red count	Protein	N.P.N.	Urea	B'	HCO_3'	Cl'	R'
	kg.	*gm.*	*cc.*	*per cent*		*per cent*	*mg. per 100 cc.*	*mg. per 100 cc.*	*cc. 0.1 N 100 cc.*	*cc. 0.1 N 100 cc.*	*cc. 0.1 N 100 cc.*	*cc. 0.1 N 100 cc.*
Operation		0				9.4			175	26	105	44
1	20.4	0	460									
2	20.0	624	1440									
3	20.2	567	1120									
4	20.2	454	800									
5	20.0	340	640							25		
6	19.7	1247	1280									
7	19.7	623	1120									
8	19.5	567	960	36	5,300,000				175	22	101	52
9	18.8	737	960									
10	18.7	680	960	41	6,910,000	9.4			161	19	98	44
11	18.2	680	960									
12	17.5	170	[illegible]	44	6,570,000	11.6			158	12	96	50
13 a.m.	16.8	170	0	47	6,480,000	12.0			160	12	93	55
p.m.							220	187		12	94	
*14 a.m.	15.8	0	20	44	6,820,000	12.1			159		93	
p.m.				47	7,890,000	11.1	315		161	7	87	67
**15 a.m.	16.1	0	20	47	7,880,000	9.8	290		166	7	103	56
p.m.				43	7,100,000	8.8	325	285	167	17	104	46

* 2000 cc. 0.9 per cent NaCl solution injected into peritoneal cavity.

** 400 cc. 2 per cent $NaHCO_3$ solution injected into femoral vein.

of stomach secretions. Dehydration of the plasma is indicated by the increase of plasma protein, the rise in red count, and the hematocrit readings (see Table II). Elman and McCaughan (5) in their much

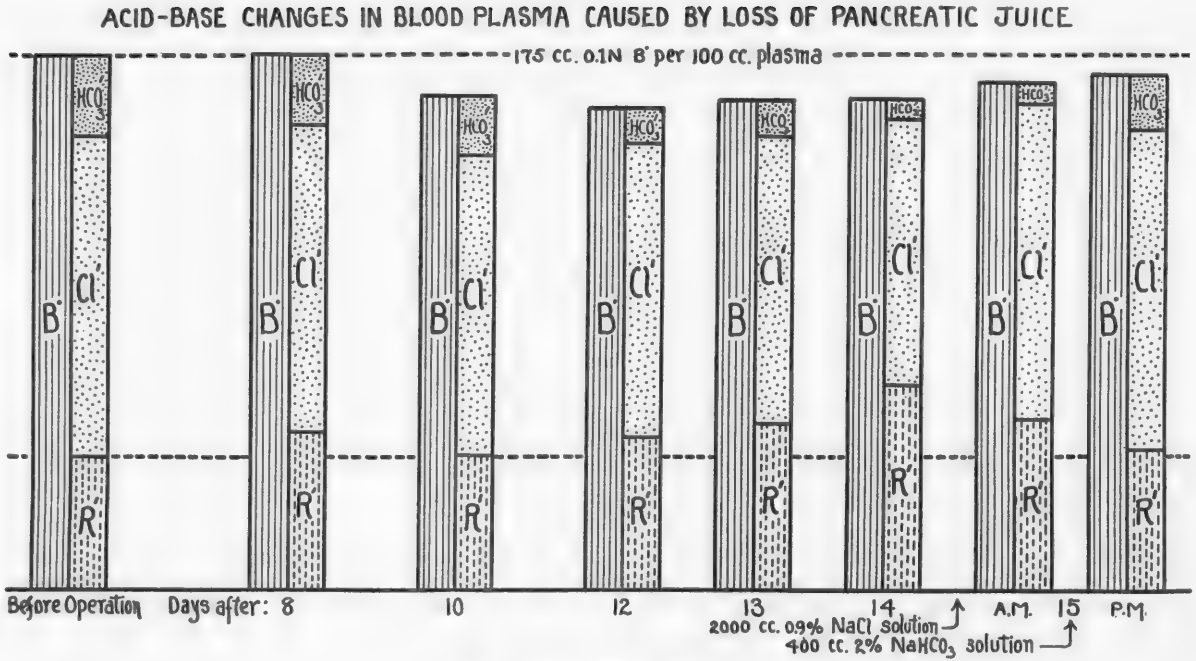

FIG. 1. From data in Table II.

more extensive series of experiments report dehydration of the blood as an outstanding finding. The few measurements of non-protein nitrogen and of urea nitrogen obtained in Experiment II near the end of the survival period show a very large accumulation of protein end-products in the plasma.

A chief purpose of these experiments was to define the changes in the acid-base structure of the plasma caused by loss of a digestive secretion containing much more of fixed base than of chloride ion. Since, when stomach secretions are lost, the relatively much larger withdrawal of chloride ion causes an extension of bicarbonate, it might be expected that loss of pancreatic juice would produce a reduction of bicarbonate.[1] This change was actually found in these two experiments and its relationship to changes in other acid-base factors in the plasma is easily apparent in the diagrams constructed from the measurements obtained in Experiment II (Fig. 1). There occurs a decrease in the concentration of fixed base and an increase in the sum of the acid factors other than the bicarbonate ion and chloride ion (the value R' in the diagrams), just as has been found to occur when stomach secretions are lost. These two changes will cause a reduction of bicarbonate unless accompanied by an equivalent or more than equivalent decrease of chloride ion concentration. This latter event occurs when stomach secretions are lost and explains the bicarbonate increase. When pancreatic juice is lost there is also, according to the data here presented, a decline in chloride ion concentration but not of sufficient extent to prevent a large reduction of bicarbonate. It is of interest to note that, had the chloride ion concentration been sustained at its initial level, bicarbonate would have been entirely obliterated. These points of agreement and of contrast between acid-base changes caused by loss of stomach secretions and by loss of

[1] The sum of the acid factors in the plasma is kept equal with the sum of the base factors by adjustment of the bicarbonate ion concentration, change in any factor causing change in bicarbonate in the direction which will maintain acid-base equivalence. In the diagrams in Figs. 1 and 2, equivalence is represented by the equal height of the two columns measuring the sum of acid and of base factors respectively, and the extent of change in bicarbonate necessary to maintain equivalence in the presence of change found in other factors is clearly described.

pancreatic juice, may be readily seen by comparing the diagrams in Fig. 2, constructed from data by Gamble and Ross (3) with those in Fig. 1. These data then demonstrate the expected finding that the continued loss in pancreatic juice of fixed base in excess of chloride ion causes a reduction of plasma bicarbonate. Elman and McCaughan (5) report that in their experiments a rise in plasma pH was noted. This would of course indicate a bicarbonate ion concen-

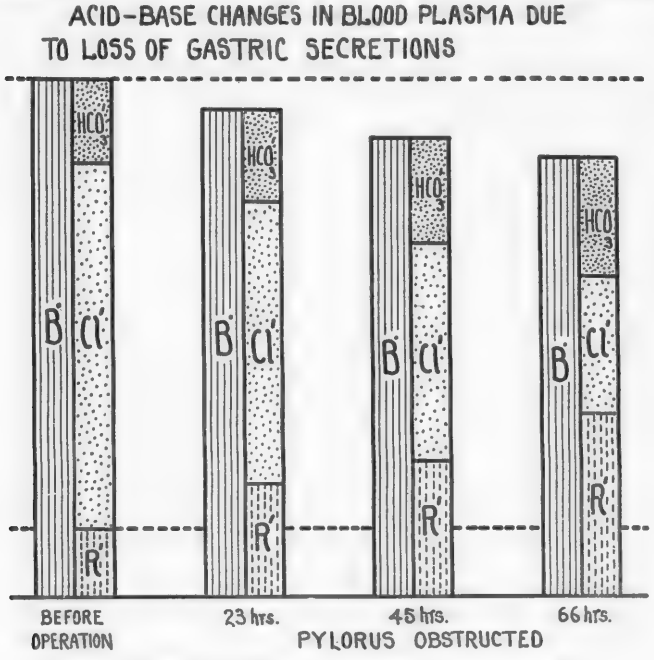

FIG. 2. From data published by Gamble and Ross (3).

tration higher than usual. They state, however, that vomiting was a regular and prominent circumstance during the last several days of the survival period, so that unquestionably there was some loss of stomach secretions as well as of pancreatic juice, an event which, as has just been noted, tends to cause extension of the plasma bicarbonate. Evidently the gist of these findings is that loss of the external secretion of the pancreas produces extensive dehydration

of the plasma and interstitial body fluids and a reduction of plasma bicarbonate in contrast with dehydration and bicarbonate extension caused by loss of stomach secretions.

Dehydration can probably be correctly regarded as the result of a loss of fixed base (3). The fixed base lost in digestive secretions is almost entirely sodium. That a large deficit of fixed base does occur from loss of stomach secretions following pyloric obstruction is easily demonstrable. This may not be directly assumed, however, as a result of withdrawal of pancreatic juice under the experimental circumstances here present for the reason that food was given from which replacement of sodium and of chloride ion can presumably to a certain extent be obtained. Pawlow's observation that ingestion of sodium bicarbonate will greatly, and a milk diet considerably, prolong the survival period obviously suits the conception of a sodium deficit. Meat, the greater part of the fixed base content of which is potassium, which cannot be used to an appreciable extent in the plasma, should be an unsuitable food. The meat given the animals in our experiments was boiled horse meat without added salt. On analysis of several samples it was found to contain per 100 gm.: K, 90 cc. 0.1 N; $Na^{\cdot}$, 11 cc. 0.1 N; and Cl', 17 cc. 0.1 N. From these data and the amounts of meat eaten by the animal in Experiment II during the 11 days preceding the appearance of symptoms and extensive changes in the blood plasma, it may be computed that the average daily intake of sodium was 65 cc. 0.1 N. We have unfortunately no measurements of the daily loss of pancreatic juice from this animal. On this point, however, the extensive data of Elman and McCaughan (5) are available. In a series of twelve experiments they found an average daily production of 250 cc. of pancreatic juice. All of their animals were smaller than the animal used in our Experiment II. This average daily quantity of juice would contain, according to the analyses in the preceding paper, about 370 cc. 0.1 N sodium. If all of the sodium in the ingested meat (65 cc. 0.1 N per day) is assumed to have been absorbed, the estimated daily deficit of sodium from the outset of the experiment is thus approximately 300 cc. 0.1 N. If the loss of this amount of sodium was accompanied by the water which contained it in the interstitial body fluids, dehydration of this animal may be

taken as having proceeded at the rate of about 200 cc. per day.[2] This is admittedly an extremely rough estimation of the probable sodium and corresponding water loss for this animal. It may be noted however that the actual loss of body weight during the first 10 days of the experimental period was approximately 2000 gm. As further evidence of sodium poverty a sample of urine obtained by catheter on the 12th day of this experiment was found to contain relatively minute amounts of sodium and of chloride, as may be seen from the following values per 100 cc. of urine: $K^{\cdot}$, 171 cc. 0.1 N; $Na^{\cdot}$, 1.6 cc. 0.1 N; Cl', 1.3 cc. 0.1 N; PO_4'', 87 cc. 0.1 N.

The experiment of attempting to prevent the appearance of symptoms by early and continued replacement of sodium was not undertaken, our chief purpose being to define the plasma changes. Attempt was however made to repair these changes, at what was apparently almost the end of the survival period, by administration of sodium chloride and sodium bicarbonate solutions. Although in both instances the condition was hopelessly advanced, repair was to a certain extent accomplished and striking though temporary improvement in behavior of the animal was observed. The dog used in Experiment I was, on the 43rd day, extremely sick, showing marked muscular weakness and apathy. Within an hour after administration of 1000 cc. of salt solution by clysis and 100 cc. of 2 per cent sodium bicarbonate solution by intravenous injection, the apathy and muscular weakness disappeared to a remarkable degree. On the following day 5 gm. of sodium bicarbonate were given in milk by stomach tube and a bread and milk diet was substituted for meat. The next morning as may be seen in Table I, there was substantial replacement of plasma base and the bicarbonate concentration was almost doubled. In Experiment II, the animal was almost *in extremis* when the administration of

[2] The concentration of sodium in the interstitial fluids is for this rough calculation taken as 150 cc. 0.1 N per 100 cc., which is the usual value found in the blood plasma of the dog. The similarity of the inorganic composition of interstitial fluids and blood plasma, except for relatively slight differences referable, in terms of the Donnan law, to unequal concentrations of protein, is indicated by recent studies of the composition of transudates and cerebrospinal fluid (6, 7). For discussion of the limitation of dehydration to interstitial fluids and blood plasma, the reader is referred to papers cited above (3).

repair materials was undertaken. On the afternoon of the 14th day there was marked prostration and respirations were of the type characteristic of severe acidosis. Salt solution, 2000 cc. of 0.9 per cent, warmed to body temperature, was injected into the peritoneal cavity. The animal survived the night and the next morning 400 cc. of 2 per cent sodium bicarbonate solution was injected into the femoral vein. By afternoon there was definite improvement in the physical behavior of the animal. The alterations of plasma factors produced by these solutions are shown in Table II and also by means of diagrams in Fig. 1. It is of interest to note that the sodium chloride solution produced an increase of fixed base and to a somewhat larger extent of chloride ion, but did not alter bicarbonate. Following the injection of bicarbonate solution there was extension of plasma bicarbonate due, curiously, to recession of R', there being only slight further increase of fixed base.

SUMMARY.

From the data given above the following explanation of the effects of continued loss of the external secretion of the pancreas may be offered. The underlying event is a steadily increasing deficit of sodium and of chloride ion due to the large requirement for these electrolytes in the construction of pancreatic juice. In consequence there is continued loss of water, chiefly from the body fluids in which sodium and chloride ion are large factors of total ionic content, *viz.*, interstitial fluids and the blood plasma. During about two-thirds of the survival period the volume and composition of the blood plasma remain approximately normal, the losses of water, sodium, and chloride ion being replaced at the expense of interstitial fluids. Reduction of the volume of these fluids is indicated by loss of body weight beginning directly after establishment of the pancreatic fistula. Ultimately reduction of plasma volume begins and, as it progresses, serious symptoms develop and death occurs unless water, sodium, and chloride ion are abundantly replaced. Owing to the relatively greater loss of sodium than of chloride ion in pancreatic juice, reduction of bicarbonate ion concentration in the plasma tends to occur. The death of the organism may be simply and reasonably explained as the result of progressive impairment of the function of the blood by the physical changes,

dehydration and acidosis, produced in the plasma by the continued loss of sodium and of chloride ion in the pancreatic juice.

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STUDIES IN EXPERIMENTAL EXTRACORPOREAL THROMBOSIS.

VII. Extracorporeal Thrombosis in Experimental Obstructive Jaundice and after the Intravenous Administration of Bile Acids.*

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Plate 20.

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The prolonged coagulation time of the blood often noted in jaundiced patients has long been recognized as of serious import in relation to surgical measures. Postoperative bleeding, a grave complication, usually occurs when there is delay in coagulation. The coagulation, calcium and prothrombin times are frequently prolonged in obstructive jaundice, particularly if the jaundice has existed for 3 or more weeks. The exact nature of the disturbance in the mechanism of coagulation of the blood, however, is unknown. Available data indicate inhibition of the formation of thrombin and partial inactivation of the thrombin already formed. The calcium and fibrinogen content of the blood, according to most investigators, is undisturbed in these cases.

Purpura in jaundice is also baffling; the coagulation time may be perfectly normal in the face of widespread subcutaneous oozing. Increased capillary permeability, probably toxic in origin, rather than

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delayed coagulation seems to be the determining factor. Both hemorrhage and purpura are sometimes terminal events in the course of jaundice. They are also encountered rather frequently in the terminal stages of certain other chronic diseases, notably in nephritis and uremia. Consequently further proof of the specific rôle of retained biliary products in the production of hemorrhage and purpura is legitimately demanded. It is possible that hemorrhage and purpura, like terminal pneumonia, may merely be expressions of impending dissolution. Such a possibility should not be overlooked.

Despite our lack of information concerning the causative mechanism of bleeding in jaundice, certain progress has been made clinically in the control of hemorrhage, postoperative oozing and purpura. Measures aimed at improvement of the patient's general condition are often followed by disappearance of the purpura. An abundance of sugar and water tends, in many cases, to increase the vigor and to decrease the surgical risk. The repeated administration of calcium chloride intravenously sometimes seems to offer protection against subsequent hemorrhage. Such measures combined tend to shorten the coagulation time of the blood and render the patient a more favorable subject for surgical intervention. If these procedures fail, there yet remains transfusion of blood, one of the most satisfactory general methods in the treatment of hemorrhage. But in a considerable number of cases, all efforts at control prove futile.

The experimental procedures were carried out in two series: (1) extracorporeal thrombosis in experimental jaundice, (a) old technic, eight experiments, (b) modified technic, ten experiments, and (2) extracorporeal thrombosis following the intravenous injection of bile acids, (a) single injections of bile acids, (b) continuous injection of bile acids with Woodyatt pump.

Extracorporeal Thrombosis in Experimental Jaundice.

The common duct of rabbits was ligated under ether anesthesia under strictly aseptic conditions. 2 to 5 days later the animals were anesthetized with tertiary trichlorobutyl alcohol, the extracorporeal loop was attached and observations made on the rate and character of formation of the thrombus. Jaundice was apparent in all of the animals on the day of experiment.

Old Technic, Eight Experiments.—The four glass sections of the extracorporeal loop were coated with paraffin but the cannulas and rubber collars were left with clean ungreased surfaces. In the normal animal circulation through the extracorporeal loop ceases in from 6 to 10 minutes, or at most, in unusually large and vigorous animals, in 25 minutes and the collodion tube is completely obstructed by a solid thrombus. If the jaundice has existed for several days the course is quite different. White thrombi composed of platelets and, later, leucocytes are laid down in about the usual manner and can be seen grossly in from 10 to 15 minutes. The formation of fibrin, however, is greatly delayed and the lumen does not become occluded for several hours. White thrombi multiply in size and numbers, chiefly on the venous side, and in some experiments pile up into cauliflower-like excrescences (Fig. 1) in the collodion tube and venous cannula so that the speed of the current is materially decreased and fibrin threads are permitted to become attached firmly. The fibrin in its turn enmeshes erythrocytes, especially in the dependent portions of the apparatus and a thin film of poorly organized thrombus results. The white thrombi in the uppermost portions of the collodion tube retain their identity much longer before being overlaid by fibrin and erythrocytes. Circulation persists in most instances for from 3 to 4 hours.

Modified Technic, Ten Experiments.—The apparatus was first assembled and then dipped in melted paraffin. Only the cannulas and the cannula collars remained unparaffined. In the normal animal with this type of preparation, circulation persists for 2 or 3 hours. White thrombi grow in the cannulas and collodion tube and after an hour or two are found overlaid by red thrombi which ultimately obstruct the lumen.

In the jaundiced rabbit the course is similar except that the flow persists for a much longer period and the white thrombi often retain their identity strikingly, without admixture of red elements or gross fibrin. Even after 3 hours of circulation the membrane may exhibit nothing but a heavy deposit of platelets and leucocytes. The thrombi tend to be laid down in a characteristic fashion being strictly localized at either end of the collodion tube so that they comprise a ring form (Fig. 2). In some experiments, the primary ring-like group of white

elements was later covered by a heavy mass of erythrocytes and fibrin which ceased growing as soon as the disparity in lumen between the glass and collodion tubes was overcome. The inner surface of the mixed thrombus was composed of a smooth fibrin sheath, practically colorless because of the washing action of the circulating blood. The reason for the peculiar localization of thrombus elements at either end of the collodion tube is obscure. It has not been observed in any experimental condition other than obstructive jaundice; probably mechanical and physical factors are responsible.

Bilirubin estimations and bile acid determinations by the quantitative Pettenkofer method of Aldrich and Bledsoe were made before the experiments were begun (Table I). During the experiment the serum bilirubin averaged 7.5 mg. and the bile acids 18.5 mg. for each 100 cc. of blood. The coagulation time was uniformly prolonged.

Type Protocol (*Modified Technic*).—The common duct was ligated August 27, 1927, and the experiment performed 4 days later when the serum bilirubin was 9.4 mg. and the level of the bile acids 17.5 mg. for each 100 cc. of blood. At 9.50 a.m. the clotting time (Lee and White method) was 9½ minutes. The extracorporeal loop with unparaffined cannulas was attached. The blood flow through the loop was brisk and the formation of eddies was marked. At the end of 10 minutes a few tiny white thrombi could be seen at the venous end of the collodion tube. At the end of 20 minutes these had grown definitely in size and numbers and a few could also be seen in the arterial end. At the end of an hour, the flow was still quite brisk. At either end of the collodion tube were many tiny white thrombi; they could not be seen in the middle portion of the tube. After 2 hours of circulation through the extracorporeal loop, the thrombi had increased in numbers and size so as to form a ring about 1 cm. wide at each end of the collodion tube. After 3 hours the ring at the venous end was distinct and white and that at the arterial end had been overlaid by red thrombi. The blood flow remained brisk and strong and apparently there was no obstruction at any point in the apparatus. The clotting time was 10 minutes.

The apparatus was detached and washed gently with a solution of sodium chloride. The collodion tube was slit longitudinally and flattened. The massed white thrombi at the venous end were creamy white, at the arterial end the red thrombus was covered by a smooth fibrin lining which was washed white by the action of the flowing blood (Figs. 3 and 4). The middle portion of the tube was free of thrombi.

Comment.—During the progress of the experiments certain facts became apparent. It was found, for example, that the condition of

the animal exerted a profound influence on the rate and character of formation of the thrombus in the loop. If the animal was vigorous and healthy and the circulation correspondingly brisk, the flow persisted for a long time and white thrombi only were deposited. On the other hand, if the animal was exhausted from long continued jaundice or was depressed by overdosage of an anesthetic, with consequent weak circulation at the beginning of the experiment, the white thrombi were soon overlaid by fibrin and erythrocytes and the lumen became occluded relatively early. Changes in the quality of the blood and variations in coagulation time appeared to exert a lesser influence than did the purely mechanical factors. For these reasons it was deemed unwise to attempt to draw too far reaching conclusions concerning specific factors. Furthermore, the course of events did not always follow the typical course anticipated. Thus in two experiments performed on animals in whom the common duct had been ligated 3 and 6 days respectively before the experiment, the results were wholly at variance with those usually encountered. In both experiments, on repeated attachment of the apparatus, the lumen became occluded in some portion within 20 minutes and large masses of loose, friable, coiled fibrin were found long before numerous white thrombi had appeared. These animals were in excellent condition and seemed to fulfill all the requirements for experimentation. In a third experiment, circulation through the loop persisted for 10 hours without occlusion. It was obviously impossible adequately to correlate such divergent results. To disregard them would be altogether unwise. That experimental obstructive jaundice and the blood changes accompanying it exert a definite influence on coagulation and the formation of thrombi in the extracorporeal loop seems proved beyond doubt, but at the same time physical and mechanical factors assume such large proportions that they approach, in importance, the purely intrahemic, chemical and physiologic abnormalities induced by the presence of jaundice. Hence in clinical jaundice the general condition of the patient as well as the results of laboratory tests of the blood would seem of great significance.

Extracorporeal Thrombosis Following the Intravenous Injection of Bile Salts.

It has been postulated at various times that the increased level of bile acids of the blood may be responsible for the prolonged coagulation time sometimes noted in cases of jaundice. Petrén, Morawitz and Bierich have maintained that such an hypothesis is untenable since bile salts added to blood *in vitro* have no effect on the coagulation time until the concentration is several times that in obstructive jaundice. Clinical studies carried on in our wards have failed to show any direct parallelism between the level of the bile acids as indicated by the Pettenkofer reaction and the coagulation time of the blood. It was thought that results of interest might be obtained by the use of the extracorporeal loop in animals that had received a solution of bile salts intravenously. Single and continuous injections of pure bile salts dissolved in physiologic sodium chloride solution were made.

Single Injection of Bile Salts.—An adequate single injection of bile salts (5 to 10 cc. of a 1 per cent solution of the sodium salts of the bile acids in physiologic sodium chloride solution) caused a definite prolongation of the coagulation time of the blood of a rabbit. As might be expected, the circulation through the loop persisted longer than normal. White thrombi were laid down in a fairly normal manner, although somewhat delayed, and after an hour or two were found overlaid by erythrocytes and small amounts of fibrin. The circulation, however, continued and there was less tendency to obstruction than occurs in the normal animal.

The rise in the level of bile acids of the blood is transitory after a single injection of pure bile salts, as has been shown by Greene and Snell in their experiments on dogs. If, as has been suggested, the duration of increase in the level of bile acids of the blood is of greater significance than the actual level attained, it would be of interest to keep animals at a constant high level of bile acids for several days and determine whether or not this factor alone could alter the coagulation time as does obstructive jaundice. A prolonged experiment of this type was not attempted, but by means of the Woodyatt pump, it was possible to approximate and maintain, for several hours, the levels of the bile acids found in clinical obstructive jaundice.

Continuous Injection of Bile Salts with Woodyatt Pump.—Five experiments were performed.

A 2.5 per cent solution of bile salts was administered in three experiments, 2 per cent in one and 6.6 per cent in the other, and specimens of blood were removed at intervals to determine the level of the bile acids (Pettenkofer value). The coagulation time was also studied and the extracorporeal loop was attached after a definite clotting disturbance was manifest. The coagulation time was not taken until about 200 mg. for each kilo of body weight had been given. At this point the coagulation time was always found to be increased, although the level of bile acids of the blood, as determined by the quantitative Pettenkofer method of Aldrich, averaged only a few milligrams higher than the normal level of 3 to 4 mg. for each 100 cc. of blood. The great quantity of cholates introduced into the circulation (200 to 300 mg. for each kilo each hour) caused hemolysis in only one experiment, in which a 6.6 per cent solution of bile salts was used. The bile acid content of the blood rose slowly the first 2 hours and reached its highest level at the close of the experiment when the animal was moribund.

The condition produced approximates in some respects that observed in jaundice. The results of the individual experiments are shown in Table II and Text-fig. 1. In Table III a general summary is tabulated. Under the conditions of these experiments from 600 to 700 mg. of bile acids for each kilo were given over a period of about 4 hours. The average coagulation time was prolonged from 3 to 15 minutes and the level of the bile acids increased from the normal of 3 to 4 mg. to an average of approximately 60 mg. at the end of the experiment. The animals became weaker and appeared to be comatose as the experiments drew to a close. Necropsy did not reveal a trace of generalized purpura or hemorrhage.[1]

In the extracorporeal loop white thrombi were never as prominent as in the normal or jaundiced animal. A few small patches of platelets could sometimes be seen grossly but as the blood pressure became

[1] In later experiments it was found that when 4 per cent solution of bile salts was injected into the marginal vein of a rabbit's ear there was a subsequent extravasation of hemoglobin or erythrocytes into the tissues surrounding the smaller venous channels. Grossly the condition seemed to be a purpuric manifestation. The vein used for the injection became thrombosed. The same results were obtained by injecting the central artery of the ear. Sellards has shown that a 5 per cent solution of bile salts injected intraperitoneally causes hemorrhagic areas and necrotic ulcers in the stomach. He could not reproduce these results by intravenous injection since the animals died immediately if a lethal dose was used. Repeated sublethal intravenous injections were precluded by venous thrombosis.

TABLE I.

Changes Observed following Ligation of the Common Duct (Series b).

Experiment No.	Days after obstruction	Average coagulation (Lee and White)	Serum bilirubin	Bile acids (Pettenkofer value)
		min.	*mg. per cent*	*mg. per cent*
Normal		3 to 4	0.0	4.0
1	5	13	7.5	13.2
2	3	13	8.7	13.4
3	4	18	6.6	10.9
4	3	18	8.1	39.0
5	5	10	3.7	16.6
6	4	10	9.4	17.5

TABLE II.

Changes Observed following Continuous Injection of Bile Salt Solution.

Experiment No.	Time	Bile salts injected	Bile acid in blood	Coagulation (Lee and White)
	hrs.	*mg.*	*per cent*	*min.*
8			3.8	3.0
	1.5	600	25.6	6.0
	2.5	1000	20.0	6.0
	3.5	1250	50.0	13.0
	4.5		27.0	15.0
9			3.9	3.0
	0.45	800	15.0	4.0
	2.00	1350	25.0	9.0
10				2.5
	1.25	270	4.9	9.0
	3.25	620	13.9	12.0
	4.25	780	22.2	15.0
	5.00	872	68.5*	15.0
11			4.1	2.5
	1.20	858	13.8	3.5
	2.20	1452	32.2	9.0
	3.20		30.0	15.0
	4.00		80.0*	

* Postmortem specimens.

progressively lower, these patches were overlaid with erythrocytes and thus when the membrane was removed, nothing but red thrombi could be seen. In one experiment this secondary sedimentation of erythrocytes did not obtain and the membrane remained practically clear except for a faint haze of tiny white thrombi and ungrouped platelets.

Type Proctocol (*Continuous Injection of Bile Salts*).—(Experiment 7). The animal used in the experiment was normal; its weight was 2600 gm. The coagulation time before the experiment was 4 minutes (Lee and White). The content of bile acids in the blood was 3.6 mg. for each 100 cc. The injection of 2.5 per cent bile salts was begun at 2.45 p.m.; at 3.50 p.m. 250 mg. had been introduced.

TABLE III.

Results of Experiments with Continuous Injection of Bile Salts.

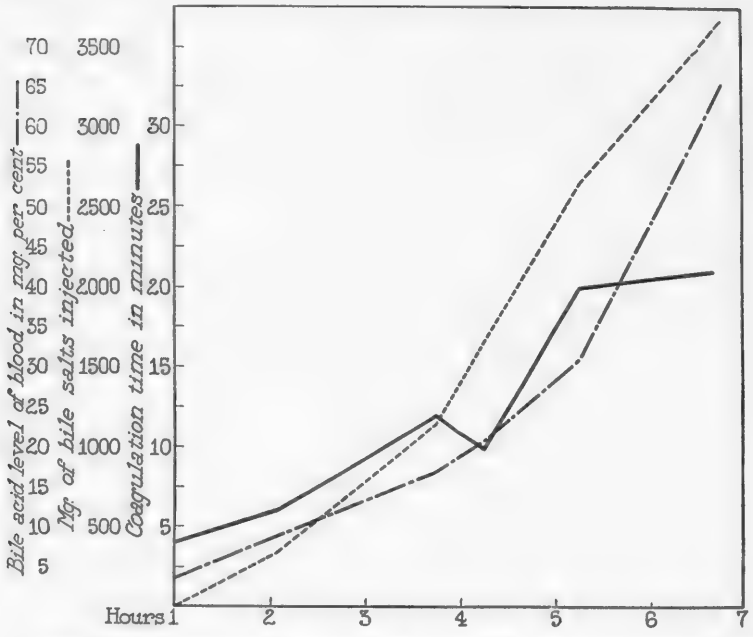
Experiment No.	Solution injected	Bile salt injected	Weight of animal	Bile salts	Duration of experiment	Coagulation		Bile acids	
						Before experiment	After experiment*	Before experiment	At end of experiment
	per cent	*mg.*	*gm.*	*mg. per kg.*	*hrs.*	*min.*	*min.*	*mg. per cent*	*mg. per cent*
7	2.5	1675	2600	640	5.5	4.5	21	3.6	65.6
8	2.5	1250	1900	658	4.5	3.0	15	3.8	50.0
9	2.5	1350	2900	466	2.0	3.0	9	3.9	25.0
10	2.0	1872	1300	670	5.0	2.5	15		68.5
11	6 6	1452	2750	537	3.5	2.5	15	4.1	80.0

* The greatest delay noted, that is at the end of the experiment usually.

The coagulation time was 6 minutes. The extracorporeal loop was attached at 3.50 p.m. At 4.20 p.m. there was an obstructing clot in the venous cannula. The apparatus was detached and cleaned. White thrombi could not be seen in the collodion tube. At 5.30 p.m. 1250 mg. had been introduced. The coagulation time was 12 minutes. Slight hemolysis was apparent. A new extracorporeal loop was attached at 5.30 p.m. At 6.00 p.m. the level of bile acids in the blood was 21 mg.; 1650 mg. had been introduced. The coagulation time was 10 minutes. White thrombi could not be seen in the apparatus. At 7.00 p.m. the level of the bile acids was 31 mg. The animal was comatose. The coagulation time was 20 minutes; 2650 mg. of bile salts had been given. At 8.30 p.m. the level of the bile acids was 65.6 mg.; 3675 mg. of bile salts had been administered. The animal was moribund. The coagulation time was 21 minutes. A few white thrombi were seen in the collodion tube. The membrane was almost clear.

Neither general hemorrhage nor purpura could be found at postmortem examination.

Text-fig. 1 illustrates the relationship between the quantity of bile salts introduced, the level of bile acids in the blood and the coagulation time in this experiment. The data from the other four experiments presented in Table II form similar curves when plotted graphically.



TEXT-FIG. 1. Relationship between the quantity of bile salts introduced, the level of bile acids in the blood and the coagulation time.

Comment.—It is interesting to speculate on the disposal of the huge excess of bile salts introduced into the circulatory system. Obviously they do not remain in the blood, for in this experiment only 21 mg. for each 100 cc. of blood were recovered after 1650 mg. had been injected. It may be assumed that the hepatic cells remove the excess bile acids rapidly and efficiently at first and then, as the injection continues, with less ease until finally death ensues. Greene and Snell in their experiments on dogs found that after a rapid single injection

of a large quantity of bile salts, there was a sudden and profound rise in the level of bile acids of the blood. This rise was short-lived and the level returned to normal within an hour or two.

DISCUSSION.

In the experiments with the Woodyatt pump the Pettenkofer value of the blood rose slowly. The injection was slow and continuous. Despite the relatively low level of bile acids at the end of the 1st hour, the coagulation time of the blood was uniformly prolonged, not markedly, yet in most instances definitely (Table II). As the injection continued, this delay in coagulability usually became progressively more marked. This raises the question of whether bile salts *in vivo* have an effect on the coagulation time of the blood which they do not exert *in vitro*.

Morawitz and Bierich, in 1906, showed that when solutions of the sodium salts of bile acids were added to samples of blood *in vitro* it was necessary to obtain a concentration of 0.5 per cent bile acids to increase the coagulation time to twice the normal value. A concentration of 0.25 of 1 per cent (250 mg. in 100 cc. of blood) did not have any appreciable effect on coagulation time. They concluded that the salts of bile acids act exactly as do neutral salts, that is, the concentration is the factor of importance and there is no specific effect. Petrén, in 1920, performed similar experiments using, however, oxalated plasma instead of whole blood. He stated that a concentration of 0.17 to 0.22 per cent was the borderline value and that 0.55 to 0.66 per cent of bile acids had a definite inhibitory effect on the coagulation of oxalated plasma. He concluded that the increased levels of bile acids found in patients with jaundice could not be responsible for "cholemic bleeding" since coagulation *in vitro* did not appear to be affected until the concentration of bile acids was several times greater than that found in jaundice. In this connection our results from the continuous injection of bile salts are of some interest. Table II and Text-fig. 1 show that the Pettenkofer value of the blood is only a little higher after the gradual injection of 200 to 400 mg. of bile salts (Experiments 7 and 10) than it was at the beginning of the experiments, yet the coagulation time is increased. In Experiments 8, 9 and 11 a much larger quantity of salts was injected during the first

hour and the Pettenkofer value is correspondingly higher; but even then the values are negligible in comparison with those of Morawitz and Bierich and Petrén. As the level of the bile acids of the blood rises during the experiments, the coagulation time is correspondingly lengthened. The highest level of the bile acids obtained (80 mg. per cent in a postmortem specimen) is well below the 0.25 per cent and 0.17 to 0.22 per cent stated by Morawitz and Bierich, and Petrén, respectively, to have a minimal effect on the coagulation of blood *in vitro*. This fact would point to the conclusion that there is some factor other than the physical concentration of bile acids which influences the clotting time in animals that have been given injections of bile salts. Petrén thought that cholemic bleeding was due to a disturbance of the function of the liver. It would seem possible that the absorption of large quantities of bile salts (such as were administered during these experiments) by the tissues, particularly the liver, might so disturb the intimate chemical and physiologic balances of the blood as to effect a delay in coagulation time. No attempt is made to dispose of the question by such an explanation but the results of these experiments would indicate that an indirect rôle of bile acids in the production of delayed coagulation time and cholemic bleeding has not yet been disproved.

An obvious criticism of our experimental methods is the large total quantity of fluid which was injected. In most instances it averaged half the calculated blood volume of the animal. This was balanced somewhat by the periodic withdrawal of blood for determinations of coagulation time and of bile acids. The effect of hemolysis which was observed definitely in one case must also be considered.

SUMMARY.

In experimental obstructive jaundice in rabbits, thrombosis is definitely delayed in the extracorporeal loop. White thrombi are laid down at the normal rate in many instances; in others there is a delay in deposition of platelets, while in still others cauliflower-like masses of platelets are rapidly deposited and tend to slow or even stop the blood stream before much fibrin is apparent. Fibrin deposits and formation of the red thrombus usually appear late in the experiment and often only at the ends of the collodion tubes. In vigorous animals,

the thrombi rarely tend to obstruct and usually cease growing when they have made up the difference between the lumen of the glass tube and of the collodion tube. On the other hand, if the animal's general condition is poor and its circulation depressed, red thrombus forms more rapidly and tends to encroach progressively on the lumen until obstruction takes place. Even in such cases circulation through the loop persists for periods much longer than in the normal animal. Judging from the gross appearance of the thrombus the quantity and quality of fibrin seem deficient.

Single injections of bile salts yield pictures similar to those seen in experimental obstructive jaundice. White thrombi are deposited but obstruction of the lumen is delayed because of inadequate formation of fibrin. The continuous intravenous injection of bile salts resulted in a progressive increase of the clotting time and in the level of bile acids of the blood. The state of the extracorporeal loop was likewise similar in many respects to that seen in obstructive jaundice. There was, however, a definite decrease in the size and numbers of white thrombi deposited on the collodion membrane.

From the evidence presented it is obvious that the processes of blood coagulation and of thrombosis in the extracorporeal loop are definitely delayed in experimental obstructive jaundice and in animals that have received intravenous injections of bile salts. No attempt is made to explain the changes found in jaundice on the basis of the increased levels of bile acids in the blood although these experiments would indicate that such a possibility has not been ruled out.

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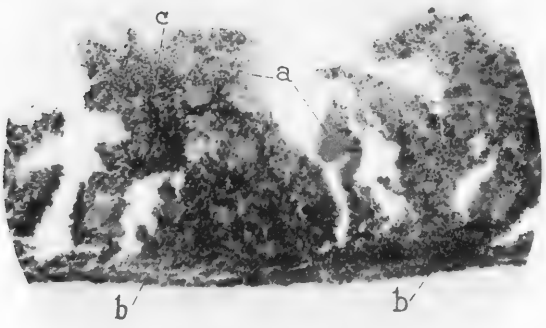
EXPLANATION OF PLATE 20.

FIG. 1. Vertical section through a thrombus mass found on collodion membrane. *a*, platelet collections; *b*, thin layer of leucocytes on the surface of collodion membrane (which was dissolved in the fixing process); *c*, red cells.

FIG. 2. Collodion tube slit longitudinally and flattened out. Heavy deposit of platelets and leucocytes at one end of tube; the remainder of tube clean and transparent.

FIG. 3. Collodion tube slit longitudinally and flattened out. Deposit of thrombi at either end of tube. *a*, washed fibrin lining of red thrombus (torn away when tube was flattened out); *b*, red thrombus; *c*, white thrombus.

FIG. 4. Cross-section of thrombus found at arterial end of tube (Fig. 3). *a*, smooth layer composed of fibrin and leucocytes; *b*, network of fibrin threads with entangled red cells and a few leucocytes; *c*, collodion membrane.



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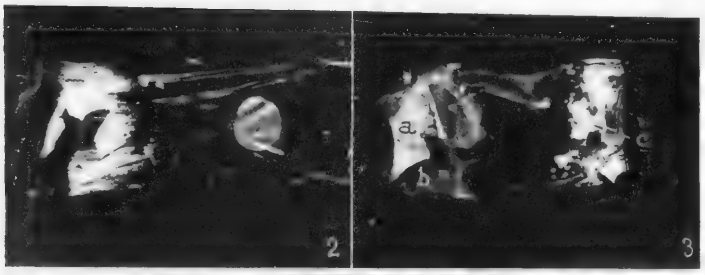

(Johnson, Shionoya, and Rowntree: Extracorporeal thrombosis. VII.)

THE PRODUCTION OF EXPERIMENTAL TYPHOID FEVER IN THE GUINEA PIG WITH AN IN VIVO PREPARED TOXIC FILTRATE OF B. TYPHOSUS.*

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Plates 21 to 23.

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The experiments of Grunbaum (1) and of Metchnikoff and Besredka (2) have demonstrated that when *Bacillus typhosus* is introduced into anthropoid apes, certain evidences of human typhoid fever can be reproduced. Positive blood cultures, agglutination reactions, fever, lesions of Peyer's patches and other features of the disease have been reproduced in this type of animal. The experiments carried out with this organism upon the smaller and more practical laboratory animals have, however, as a whole, proven unsatisfactory. Although a septicemia, peritonitis and death can be readily produced in such smaller animals, the resultant picture bears little if any relationship to the human disease.

Besredka (3) has found that smaller animals primarily prepared by feeding bile are vulnerable to the ingestion of typhoid bacilli and present a more protracted infection with certain features allied to human typhoid. More recently Sedan and Herrmann (4) have employed the injection of typhoid bacilli into the subconjunctival tissues. They have produced by this means a continued fever, diarrhea, tumefaction of Peyer's patches and certain other features analogous to human typhoid although the microscopic study does not appear identical with that found in the human infection. Gory and Dalsace (5) have also reported typhoid infection in the guinea pig, employing the method of Sedan and Herrmann. Although they state that the human disease has been duplicated, a review of their pathological study shows that multiple abscesses were produced in the liver, spleen and the lymphoid tissues of the peritoneal cavity including

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Peyer's patches. They also specify that acute cholecystitis was almost constant and acute pericarditis was at times present. Their results indicate that through the method employed they prolonged the duration of the usual septicemia, permitting thereby the formation of widespread acute exudative lesions. Such lesions do not conform with the host response characteristic of the human disease, the histopathology of which was first most fully described by Mallory (6).

In addition to numerous transmission experiments performed with the organism proper, extensive experimentation has been carried out with the toxic products. Evidence has been advanced in support both of its endotoxic and ectotoxic nature. A great variety of methods have been employed for the purpose of obtaining a specific toxin. A general review of the literature appertaining to the toxin of the organism is to be found in the work of Gay (7).

Persistent difficulty has been encountered in procuring a true specific toxin for many microorganisms, especially those regarded as endotoxic in nature. In this connection, Duval and Hibbard (8) have reported the effects produced in animals by the injection of a toxic material obtained from the streptococcus of scarlatina by means of an *in vivo* process. In their method the supply animal was first immunized by several injections of the streptococcus administered at weekly intervals. It occurred to us that the employment of the animal host in some intermediary capacity may be essential in the production of a toxic material more closely allied to that demonstrated in the typhoid toxemia of man.

When the typhoid bacillus is introduced into the peritoneal cavity of normal guinea pigs an exudative peritonitis is produced. In this conflict of the invading microorganism and the animal host the usual resultant features of an acute inflammatory reaction are found. It appeared likely that in this field of activity in which many factors both of host and invader have been put into action, a toxic substance might be produced which when injected into animals would yield a different character of response than that obtained by the living microorganism.

The experiments herein reported demonstrate the results obtained by the injection into guinea pigs of the toxic factor procured through this *in vivo* method.

EXPERIMENTAL.

A peritonitis was produced in guinea pigs by the inoculation of cultures of *B. typhosus*. The virulence of the strain was exalted by passage through several generations of guinea pigs so that within 7 to 10 hours after injection the animals became very sick and the abdomen distended with exudate. The animals were

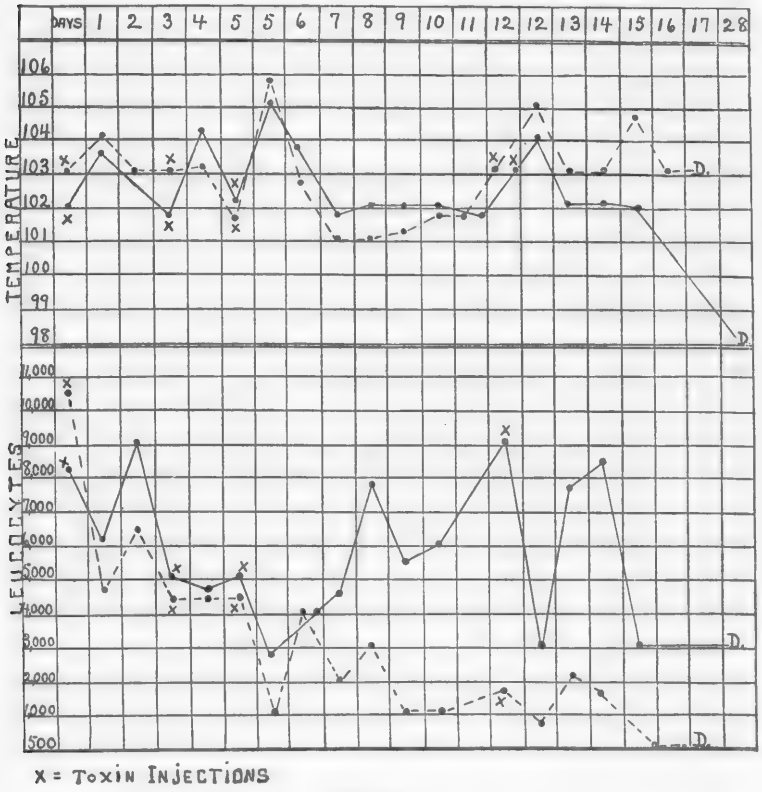

CHART 1.

then sacrificed and the fluid was withdrawn from the peritoneal cavity. The exudate varied from a slightly smoky yellow liquid to one of a cloudy, flaky character, rich in cellular elements. This material was diluted to approximately four times its volume with sterile water and filtered through an N Berkefeld filter. The resultant bacteria-free filtrate was employed for animal injection. It was found preferable to employ freshly prepared filtrate at each interval injection.

Twenty-four guinea pigs have thus far been employed. The protocols of six representative animals of this group have been selected. Of these six, two received the injections subcutaneously, two intraperitoneally and two intracardially. All six animals died in approximately 2 to 4 weeks. The autopsy findings and microscopic study are described under pathology.

Experiment 1.—Two guinea pigs weighing approximately 250 gm. were given four subcutaneous injections of *in vivo* prepared toxin as follows: Each received 4 cc. as a primary injection, 3 cc. 3 days later, 5 cc. on the 5th day and 4 cc. on

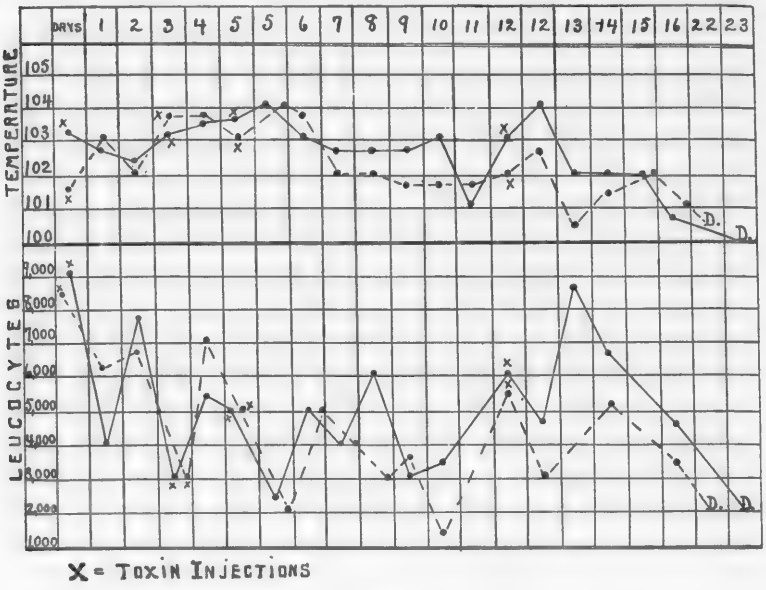

Chart 2.

the 12th day. One of these animals died on the 17th day and the other 11 days later. The temperature and leucocytic counts of each are shown in Chart 1.

As can be seen, subsequent to the injections there usually occurred a rise in temperature and a drop in the leucocytic count. One of the animals showed a drop of the count to 500 cells and the other to 3000 cells per c. mm.

Both animals gradually lost in weight and their appetite became poor. No other clinical manifestations were observed.

Experiment 2.—Two guinea pigs were given four intraperitoneal injections of *in vivo* prepared toxin on the same days as those of Experiment 1 but the following respective amounts were administered, 2 cc., 2 cc., 3 cc. and 4 cc.

The febrile and leucocytic reactions in these two animals were quite consistent and closely parallel. The leucocytic count in both instances dropped to nearly 2000 cells per c. mm. with frequent counts of around 3000 as can be seen in Chart 2. These animals lost in weight, one showing considerable emaciation. One animal died on the 22nd and the other on the 23rd day following the inoculations. No diarrhea was observed in either animal.

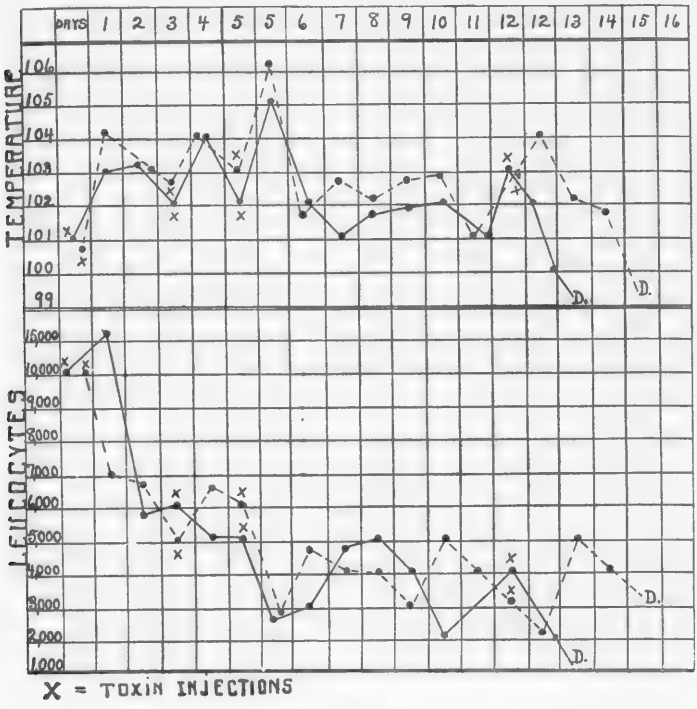

CHART 3.

Experiment 3.—Two guinea pigs were inoculated intracardially with the *in vivo* prepared typhoid toxin. They each received on the 1st day, 1 cc. of the toxin, on the 3rd day, 1 cc. of the toxin, on the 5th day, 2 cc., and on the 12th day, 1.5 cc. Their febrile response and leucocytic decline were marked and followed constantly the injections. The fever rose as high as 106.4°F. for one animal and 105°F. for the other. The leucocytes dropped below 3000 per c. mm. on several occasions, the original counts before injection being approximately 10,000 cells per c. mm. One of these animals died on the 13th day and the other

on the 15th day. The reactions by this route of inoculation were sharper (see Chart 3) and the animals died more quickly.

It can be seen from a review of the charts of these six animals that as a whole the clinical records are analogous in character. With each injection of the toxic material, irrespective of the route of administration, a rise of temperature and a drop of the leucocytes occurred. The febrile response was of a transient character lasting for 2 or 3 days after each inoculation. The abatement of the fever is attributable most likely to the fact that no continued toxemia or accumulative toxic action existed since no living virus was injected. In the instance of the leucocytes, however, after the first injection of toxin the total count seldom returned to the normal; the leucopenia continued and became accentuated subsequent to each inoculation. It would appear that the particular factors or mechanism essential for the production of leucopenia was continued over a longer period of time than the phenomena producing the fever. In no instance was diarrhea observed and aside from loss of appetite and weight and progressive weakness together with the leucocytic and febrile response no other clinical features were discernible.

Pathology.

The postmortem examination of the animals in which death followed the typhoid toxin inoculations showed as a whole the same general gross picture. In certain of the animals the changes were more accentuated than in others but this variation was only in degree or extent and not in the character of the lesions.

At the inoculation site of the animals injected subcutaneously there was found a congested and edematous area about 2 to 3 cm. in diameter. Small hemorrhagic extravasations were at times noted.

The abdominal cavity showed the lymphatic glands to be greatly enlarged and in some of the glands marked congestion and hemorrhage were present. (See Fig. 1.)

Spleen.—This structure was usually of a deep red color and moderately increased in size. The consistence was soft and the pulp stripped off readily, although two of the six spleens were quite firm. No evidences of miliary abscess formation or areas of necrosis were found.

Intestines.—The examination of the intestinal tract showed throughout a

marked enlargement of all lymphoid structures. The solitary follicles protruded well into the lumen and were frequently congested. The Peyer's patches were greatly elevated (see Figs. 2 and 3), often reddened and at times showed slight ulceration. Occasionally a marked necrosis and ulceration of the patch occurred. The fecal content in these areas was fluid in character but no blood was observed.

Liver.—This organ was enlarged, congested and friable and of a deep red color. Scattered throughout the structure were found yellow focal areas varying in size from 1 to 3 mm. The gall bladder showed nothing of note.

Kidneys and Adrenals.—These structures were somewhat swollen and the vessels showed considerable congestion. Evidences of yellowish discolorations suggestive of degeneration were sometimes found.

Bone Marrow.—This was more deeply reddened than normal marrow of the guinea pig.

Heart and Lungs.—No gross lesions were observed. In no instance was pneumonia found present.

Nothing else of note was observed in the gross study of other structures of the body.

Microscopic.—The voluntary muscle in the region of the subcutaneous inoculation showed hyaline or waxy necrosis of this structure. Scattered wandering monocytes or "endothelial" cells were seen about the necrotic muscle. The enlargement of the lymphatic glands especially of the peritoneal cavity and of the solitary follicles and Peyer's patches was found to be due to the hyperplasia of the lymphoid structures and to the accumulation of "endothelial" cells. Many "phagocytic cells" of Mallory were noted in which the engulfed elements consisted of portions of lymphoid cells, nuclear fragments and at times erythrocytes. In certain of the lymphoid structures marked congestion, hemorrhagic extravasations and areas of necrosis were seen. Some of the Peyer's patches showed a loss of the mucosa and necrosis of the cellular elements of the enlarged nodule with destruction of the muscularis mucosæ. (See Figs. 4 to 6.) Sections of the spleen demonstrated marked congestion of the pulp and in some of the animals, hemorrhages were found in this structure. Escape of the hemoglobin was noted in masses of the erythrocytes presenting thereby shadow or phantom corpuscles. (See Fig. 7.) Scattered throughout the splenic structure but particularly in the pulp were endothelial cells many of which showed phagocytosis especially of the shadow red cells.

The liver sections revealed degenerative changes including areas of focal necrosis. These necrotic areas showed no special zonal location occurring centrally or in the portal areas or extending through both zones, and they varied in their size and extent. In some of these areas hemorrhage was present and many of the extravasated cells were located within the protoplasm of the phagocytic cells. The smaller areas of focal necrosis showed replacement of the hepatic cells by the endothelial type of cell. (See Fig. 8.)

The kidneys showed congestion of the vessels and degenerative changes in the

epithelium of the uriniferous tubules. The bone marrow showed marked congestion and at times hemorrhagic extravasations. Nothing noteworthy was found in other structures examined. It is of interest to note that no polymorphonuclear neutrophils or other elements of an acute exudative inflammation were found in the various lesions studied.

Controls.—In a previous article (9) we reported parallel experiments in which *B. coli communior* was employed. Fifteen guinea pigs were used and the results obtained therein were unlike those obtained with *B. typhosus*. Evidences of toxemia with degenerative and hemorrhagic lesions were found. Although death was produced by the toxin of this microorganism, the gross and microscopic aspect did not resemble the human typhoid lesion.

In this connection, the paratyphoid epizootics occurring in the guinea pig should be mentioned. The lesions of this infection as observed by one of us (10) and as studied by Howell and Schultz (11) do not resemble the lesions herein described. Work upon the effects in guinea pigs of the toxic material prepared in a similar manner from paratyphoid bacilli is under way.

DISCUSSION.

In general microorganisms belonging to the so called endotoxic group do not yield *in vitro* a satisfactory specific toxin. This has greatly interfered with the progress of the study of the true nature and effects of such microorganisms. For example, the preparation of a specific toxin from the streptococcus considered as the cause of scarlet fever has been attended with difficulties. Dochez (12) considers that this microorganism contains at least two varieties of poisons. Duval (8), on the other hand, employing his *in vivo* method regards the toxin procured by him from the streptococcus of scarlatina as the true specific toxin of this microorganism. In the instance of the typhoid bacillus, the same general difficulty of obtaining a specific poison has been encountered although numerous and diverse methods have been applied.

A definite toxemia is manifestly present in typhoid fever and the toxin produces in the human host a specific pathological picture. It is not improbable that such a microorganism forms its specific

toxin only when invading its natural host, in other words during its function of pathogenesis. A different or more complete biological process may be evolved *in vivo* as contrasted with *in vitro* activity. Again the somatic cells of the invaded host may play some essential rôle in the production of the specific pathogenic toxin. Because of these possibilities of a differential nature of such toxins, the *in vivo* method was employed. It is believed that the toxic material obtained through this process closely simulates in its action on the inoculated host the activities of the typhoid toxin as evidenced in the human disease.

SUMMARY.

When the typhoid bacillus is injected into the peritoneal cavity of guinea pigs acute peritonitis and death are produced. The character of the exudate is variable as to the elements present but is usually of a serous type with slight clouding due to the presence of polymorphonuclear neutrophils, mononuclear cells and bacteria. When the Berkefeld filtrate of this exudative material is inoculated into normal guinea pigs either subcutaneously, intraperitoneally or intracardially, the character of response obtained on the part of the host is quite at variance with that produced by the inoculation of the living typhoid bacillus. A febrile reaction and marked leucopenia, as a rule, are persistent and are accentuated after each injection the latter often reaching below 1000 cells per c. mm. There is a loss of weight of a variable extent in all animals and in some the emaciation is extreme. The animals were given four such inoculations and all succumbed in from 2 to 4 weeks. The intracardiac route produces death more quickly and the reactions are more clear-cut when this route is employed. At autopsy a general tumefaction and congestion of the lymphoid structures more especially of the abdominal cavity are found. Peyer's patches and the solitary follicles of the intestinal tract are likewise involved and in some of the patches slight ulceration is noted; occasionally, there occur extreme ulceration and necrosis of the patch. The spleen is enlarged and usually softened. Microscopically, marked endothelial cell proliferation is noted especially in the lymphoid structures and in many instances the phagocytic cells of Mallory are found. These cells include within their

cytoplasm elements of the surrounding structures. In the spleen there are present congestion, and hemorrhages with many "shadow" red blood cells. Phagocytosis of the red cells by the endothelial cells is present. In the liver, areas of focal necrosis are found and phagocytic cells are seen. In the animals inoculated subcutaneously, localized degenerative changes are observed especially in the muscular structures.

From these results it can be seen that the reactions and injury of the animal body by the toxic filtrate employed, are quite similar to the changes produced by the specific toxin in human typhoid fever.

CONCLUSION.

During the activity of peritonitis produced in the guinea pig by means of *Bacillus typhosus*, there is formed in the exudative material a filtrable toxic moiety which when inoculated into normal animals of this species, produces certain of the clinical phenomena and a pathological picture simulating that of human typhoid fever.

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EXPLANATION OF PLATES

PLATE 21.

FIG. 1. Lesions of mesenteric lymph glands. These structures are greatly increased in size; congestion and hemorrhage are at times present.

FIG. 2. Specimen of a portion of the ileum showing Peyer's patch with enlargement and elevation of the structure and early ulceration at the upper portion. (Enlarged for detail.)

FIG. 3. Lesion similar in character to that in Fig. 2, with somewhat more marked swelling.

FIG. 4. Lesion of Peyer's patch showing marked swelling of the patch, and in certain areas, there is a loss of the epithelial lining. (Low power.)

PLATE 22.

FIG. 5. Cellular elements of swollen Peyer's patch. Cells of the phagocytic type of Mallory, which contain lymphoid cells and nuclear fragments, can be seen. (High power.)

FIG. 6. High power of lesion of the lymphatic gland, demonstrating the character of the proliferating cells.

PLATE 23.

FIG. 7. Section of spleen showing congestion and hemorrhage. The erythrocytes for the most part are of the "shadow cell" type (loss of hemoglobin) and some are engulfed by phagocytes.

FIG. 8. Area of focal necrosis in the liver. The hepatic cells have been replaced by the endothelial cells which have phagocytized fragments of red blood cells and nuclear portions. (High power.)

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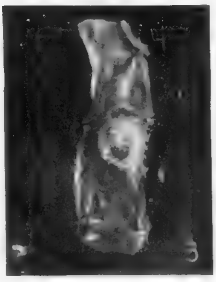

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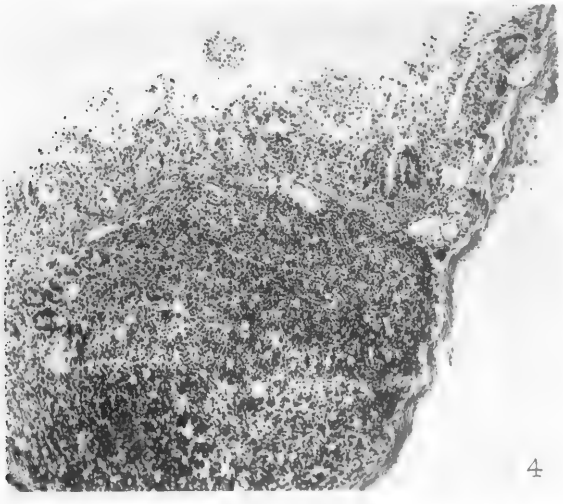

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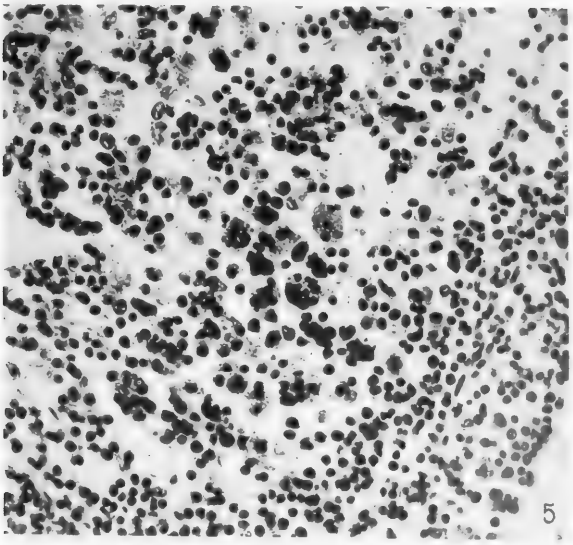

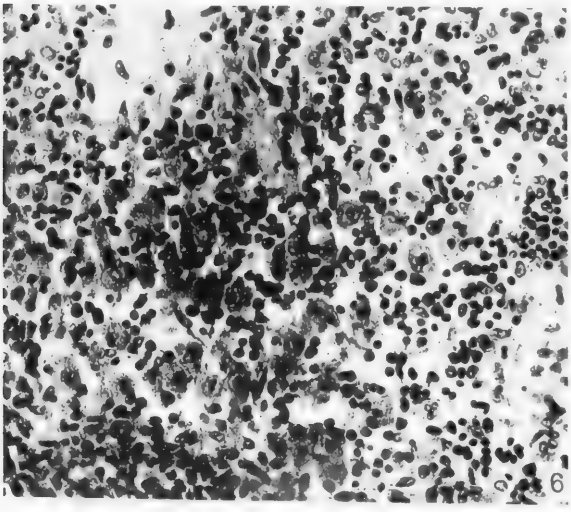

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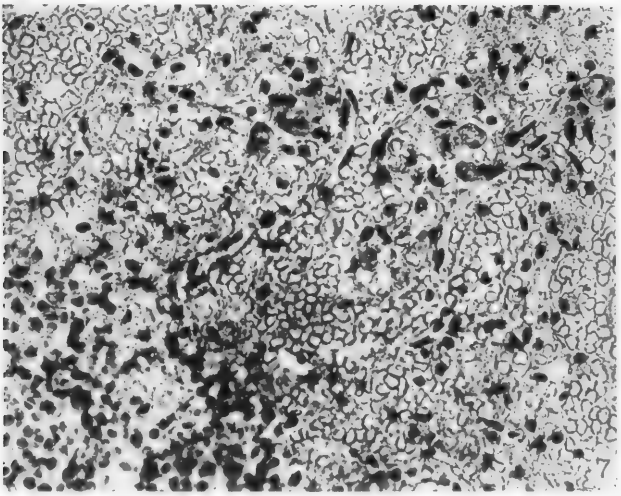

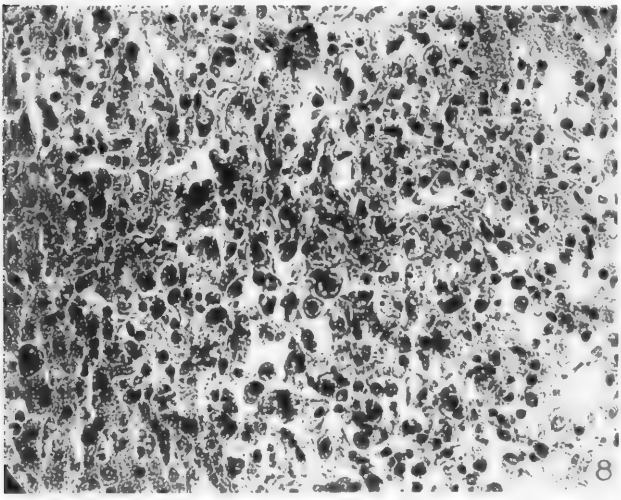

(Harris and Larimore: Experimental typhoid fever.)

INDEX TO AUTHORS.

B

Baker, Lillian E., and **Carrel, Alexis.** Nitrogen metabolism of normal and sarcomatous fibroblasts in pure cultures, 533

Barach, Alvan L. Factors involved in the production of immunity with pneumococcus vaccine. I. Active and passive immunity during the first seven days after injection of antigen, 83

Barron, E. S. Guzman. See HARROP and BARRON, 207

Bauer, Johannes H., and **Hudson, N. Paul.** The incubation period of yellow fever in the mosquito, 147

Bronfenbrenner, J. See HETLER and BRONFENBRENNER, 269

Brown, Wade H. Influence of light environment on the growth and nutrition of normal rabbits with especial reference to the action of neon light, 31

—. Influence of light environment on the growth of hair in normal rabbits with especial reference to the action of neon light, 57

—. Influence of light environment on the organic constitution of normal rabbits with especial reference to the action of neon light, 567

C

Carrel, Alexis, and **Ebeling, Albert H.** The fundamental properties of the fibroblast and the macrophage. III. The malignant fibroblast of Sarcoma 10 of the Crocker Foundation, 105

Carrel, Alexis, and **Ebeling, Albert H.** The fundamental properties, of the fibroblast and the macrophage. IV. The malignant fibroblast of Jensen sarcoma, 285

—. See BAKER and CARREL, 533

Christie, Ronald V. An experimental study of diathermy. VI. Conduction of high frequency currents through the living cell, 235

E

Earle, W. R. Studies upon the effect of light on blood and tissue cells. I. The action of light on white blood cells *in vitro*, 457

—. II. The action of light on erythrocytes *in vitro*, 667

—. III. The action of light on fibroblasts *in vitro*, 683

Ebeling, Albert H. See CARREL and EBELING, 105, 285

Eliot, Calista P. See FORD and ELIOT, 475

F

Ford, William W., and **Eliot, Calista P.** The transfer of rat anemia to normal animals, 475

G

Gamble, James L., and **McIver, Monroe A.** The acid-base composition of gastric secretions, 837

— and —. Acid-base composition of pancreatic juice and bile, 849

Gamble, James L., and **McIver, Monroe A.** Body fluid changes due to continued loss of the external secretion of the pancreas, 859

Goodner, Kenneth. Experimental intradermal pneumococcus infection in rabbits, 1

—. Further experiments with the intradermal pneumococcus infection in rabbits, 413

Gowen, John W. See SHOPE and GOWEN, 21, 25

H

Haden, Russell L., and **Orr, Thomas G.** The blood chlorides in proteose intoxication, 639

— and —. Chemical findings in the blood of the dog after temporary obstruction of the pylorus, 591

— and —. The effect of sodium chloride on the chemical changes in the blood of the dog after obstruction of the cardiac end of the stomach, 627

—. See ORR and HADEN, 339

Hammett, Frederick S., and **Wallace, Vilma L.** Studies in the biology of metals. VII. The influence of lead on the development of the chick embryo, 659

Harnes, Alvin R. Biometry of calcium, inorganic phosphorus, cholesterol, and lipoid phosphorus in the blood of rabbits. I. Normal animals from recently acquired stock, 549

Harris, William H., and **Larimore, Ogilvie M.** The production of experimental typhoid fever in the guinea pig with an *in vivo* prepared toxic filtrate of *Bacillus typhosus*, 885

Harrop, George A., Jr., and **Barron, E. S. Guzman.** Studies on blood cell metabolism. I. The effect of methylene blue and other dyes upon the oxygen consumption of mammalian and avian erythrocytes, 207

Harvey, Harold D. See MELENEY, ZAYTZEFF, HARVEY, and ZAU, 299

Haselbauer, Peter. See STEWART and HASELBAUER, 449

Hetler, D. M., and **Bronfenbrenner, J.** Studies on the bacteriophage of d'Herelle. IX. Evidence of hydrolysis of bacterial protein during lysis, 269

Hitchcock, Charles H. Studies on indifferent streptococci. I. Separation of a serological group—Type I, 393

—. II. Observations on the distribution of indifferent streptococci in the throats of rheumatic and non-rheumatic individuals, 403

Howard, Marion. See BROWN, 31, 57, 567

Hudson, N. Paul. See BAUER and HUDSON, 147

J

Johnson, Walter R., Shionoya, Takuji, and **Rowntree, Leonard G.** Studies in experimental extracorporeal thrombosis. VII. Extracorporeal thrombosis in experimental obstructive jaundice and after the intravenous administration of bile acids, 871

Jones, F. S. Agglutination by precipitin. II, 183

K

Korb, Charles. See MUCKENFUSS and KORB, 277

L

Lancefield, Rebecca C., and **Todd, E. W.** Antigenic differences between matt hemolytic streptococci and their glossy variants, 769

—. See TODD and LANCEFIELD, 751

Landsteiner, K., and **Levine, Philip.** On the inheritance of agglutinogens of human blood demonstrable by immune agglutinins, 731

— and **van der Scheer, J.** Serological differentiation of steric isomers, 315

Larimore, Ogilvie M. See HARRIS and LARIMORE, 885

Levine, Philip. See LANDSTEINER and LEVINE, 731

Long, Perrin H., Olitsky, Peter K., and **Stewart, Fred W.** The rôle of streptococci in experimental poliomyelitis of the monkey, 431

—. See OLITSKY and LONG, 199, 379

Lurie, Max B. The fate of human and bovine tubercle bacilli in various organs of the rabbit, 155

M

Marmorston-Gottesman, J., and **Perla, David.** Immunological studies in relation to the suprarenal gland. III. The effect of injections of epinephrine on the hemolysin formation in normal rats, 225

Marsh, Pauline. See GAMBLE and MCIVER, 837, 849, 859

Matteson, Elizabeth. See GAMBLE and MCIVER, 837

McIver, Monroe A. See GAMBLE and MCIVER, 837, 849, 859

Meleney, Frank L., Zaytzeff, Helen, Harvey, Harold D., and **Zau, Zung-Dau.** Reciprocal agglutination and absorption of agglutinin tests with fifty-four strains of hemolytic streptococci associated with an epidemic of puerperal fever, 299

Meleney, Henry Edmund. Relapse phenomena of *Spironema recurrentis*, 65

—. Suppression of the first attack with subsequent relapse: an immune phenomenon in experimental relapsing fever, 805

Muckenfuss, Ralph S. Studies on the bacteriophage of d'Herelle. XI. An inquiry into the mode of action of antibacteriophage serum, 709

—. XII. Concerning the production of phage from bacterial cultures, 723

— and **Korb, Charles.** Studies on the bacteriophage of d'Herelle. X. Toxin production by normal and by phage-resistant Shiga dysentery bacilli, 277

Mueller, J. Howard. The effect of oxidation of filtrates of a chicken sarcoma (Chicken Tumor I—Rous), 343

N

Nelson, John B. Observations on flagellar and somatic agglutination, 811

—. The removal of agglutinin from sensitized motile bacteria, 825

—. Studies on a paratyphoid infection in guinea pigs. V. The incidence of carriers during the endemic stage, 647

Noguchi, Hideyo. Etiology of Oroya fever. XIII. Chemotherapy in experimental *Bartonella bacilliformis* infection, 619

—. The etiology of trachoma, suppl. 2, pp. 1–53

O

Olitsky, Peter K., and **Long, Perrin H.** The action of the Levaditi strain of herpes virus, and of vaccine virus in the guinea pig. Single and combined effects, 379

— and —. The relation of streptococci to herpes virus encephalitis, 199

—. See LONG, OLITSKY, and STEWART, 431

Orr, Thomas G., and **Haden, Russell L.** Chemical changes in the blood of the dog in experimental peritonitis, 339

—. See HADEN and ORR, 591, 627, 639

P

Pappenheimer, Alwin M. See PARKER and PAPPENHEIMER, 695

Parker, Julia T., and **Pappenheimer, Alwin M.** Experimental pneumonia in guinea pigs. I. The effect of certain toxic autolysates of pneumococci, 695

Pearce, Louise. Reciprocal effects of concomitant infections. II. The influence of vaccinal immunity on the reaction to experimental syphilis, 125

—. III. The influence of vaccinia and of vaccinal immunity on the reaction to infection with experimental syphilis (intracutaneous inoculation), 363

Perla, David. See MARMORSTON-GOTTESMAN and PERLA, 225

R

Ramsdell, Susan Griffith. Surface tension of sera as affected by the precipitin reaction, 615

Reznikoff, Paul. The effect of some of the chemical constituents of tubercle bacilli on the protoplasm of *Amœba dubia*, 193

Rivers, Thomas M., and **Stewart, Fred W.** Virus III encephalitis, 603

Robertson, Oswald H. See SIA, ROBERTSON, and WOO, 513

Rowntree, Leonard G. See JOHNSON, SHIONOYA, and ROWNTREE, 871

S

Shionoya, Takuji. See JOHNSON, SHIONOYA, and ROWNTREE, 871

Shope, Richard E. The influence of cholesterol on experimental tuberculosis, 321

— and **Gowen, John W.** Cholesterol and cholesterol ester content of bovine colostrum, 21

— and —. Cholesterol and cholesterol ester in the blood serum of cattle late in pregnancy and during the early lactation period, 25

Shwartzman, Gregory. Studies on *Bacillus typhosus* toxic substances. I. Phenomenon of local skin reactivity to *Bacillus typhosus* culture filtrate, 247

Sia, Richard H. P., Robertson, Oswald H., and **Woo, Shutai T.** A study of the mechanism of recovery from lobar pneumonia, 513

Sloan, Dorothy. See GAMBLE and McIVER, 859

Smith, Theobald. The relation of the capsular substance of *Bacillus coli* to antibody production, 351

Soroka, Max. See BARACH, 83

Stewart, Fred W., and **Haselbauer, Peter.** Virus neutralization experiments with Rosenow's and Pettit's antipoliomyelitic sera, 449

—. See LONG, OLITSKY, and STEWART, 431

—. See RIVERS and STEWART, 603

T

Tillett, William S. Active and passive immunity to pneumococcus infection induced in rabbits by immunization with R pneumococci, 791

Todd, E. W. The conversion of hemolytic streptococci to non-hemolytic forms, 493

Todd, E. W., and **Lancefield, Rebecca C.** Variants of hemolytic streptococci; their relation to type-specific substance, virulence, and toxin, 751

—. See LANCEFIELD and TODD, 769

Tolman, Maurice M. See GAMBLE and McIVER, 849

V

van der Scheer, J. See LANDSTEINER and VAN DER SCHEER, 315

W

Wallace, Vilma L. See HAMMETT and WALLACE, 659

Woo, Shutai T. See SIA, ROBERTSON, and WOO, 513

Z

Zau, Zung-Dau. See MELENEY, ZAYTZEFF, HARVEY, and ZAU, 299

Zaytzeff, Helen. See MELENEY, ZAYTZEFF, HARVEY, and ZAU, 299

INDEX TO SUBJECTS.

A

Acid (s):
-base composition of gastric secretions (GAMBLE and MCIVER) 837
— — — pancreatic juice and bile (GAMBLE and MCIVER) 849
Bile, extracorporeal thrombosis after intravenous administration (JOHNSON, SHIONOYA, and ROWNTREE) 871

Adrenal:
Immunological studies. III (MARMORSTON-GOTTESMAN and PERLA) 225

Agglutination:
Flagellar and somatic (NELSON) 811
Hemolytic streptococci associated with epidemic of puerperal fever studied by reciprocal agglutination and absorption of agglutinin tests (MELENEY, ZAYTZEFF, HARVEY, and ZAU) 299
Precipitin. II (JONES) 183

Agglutinin:
Hemolytic streptococci associated with epidemic of puerperal fever studied by reciprocal agglutination and absorption of agglutinin tests (MELENEY, ZAYTZEFF, HARVEY, and ZAU) 299
Immune, demonstrating inheritance of agglutinogens of human blood (LANDSTEINER and LEVINE) 731

Agglutinin—*continued:*
Removal from sensitized motile bacteria (NELSON) 825

Agglutinogen:
Blood, human, inheritance demonstrable by immune agglutinins (LANDSTEINER and LEVINE) 731

Amœba:
dubia, effect of chemical constituents of tubercle bacilli on protoplasm (REZNIKOFF) 193

Anemia:
Rat, transfer to normal animals (FORD and ELIOT) 475

Antibacteriophage serum:
See Serum.

Antibody:
Bacillus coli, relation of capsular substance (SMITH) 351

Antigen:
Pneumococcus, active and passive immunity after injection (BARACH) 83
Streptococci, antigenic differences between matt hemolytic forms and their glossy variants (LANCEFIELD and TODD) 769

Antipoliomyelitic serum:
See Serum, poliomyelitis.

Autolysate:
Pneumococcus, toxic (PARKER and PAPPENHEIMER) 695

B

Bacillus:
coli, relation of capsular substance to antibody production (SMITH) 351

Bacillus—*continued:*
dysenteriæ, Shiga, toxin production by normal and by phage-resistant strains (MUCKENFUSS and KORB) 277
tuberculosis, effect of chemical constituents on protoplasm of *Amœba dubia* (REZNIKOFF) 193
—, human and bovine, fate in organs of rabbit (LURIE) 155
typhosus, local skin reactivity to culture filtrate (SHWARTZMAN) 247
—, toxic filtrate prepared *in vivo*, producing typhoid fever (HARRIS and LARIMORE) 885
—, — substances. I (SHWARTZMAN) 247
Bacteriophage:
Production from bacterial cultures (MUCKENFUSS) 723
Toxin production by normal and by phage-resistant Shiga dysentery bacilli (MUCKENFUSS and KORB) 277
Twort-d'Herelle phenomenon. IX (HETLER and BRONFENBRENNER) 269
— —. X (MUCKENFUSS and KORB) 277
— —. XI (MUCKENFUSS) 709
— —. XII (MUCKENFUSS) 723
Bacterium:
Culture, production of bacteriophage (MUCKENFUSS) 723
Protein, hydrolysis during lysis (HETLER and BRONFENBRENNER) 269
Sensitized motile, removal of agglutinin (NELSON) 825
Bartonella:
bacilliformis, infection, chemotherapy (NOGUCHI) 619
Base:
Acid-, composition of gastric secretions (GAMBLE and MCIVER) 837
—, — — pancreatic juice and bile (GAMBLE and MCIVER) 849
Bile:
Acid-base composition (GAMBLE and MCIVER) 849
Acid, extracorporeal thrombosis after intravenous administration (JOHNSON, SHIONOYA, and ROWNTREE) 871
Bird:
Erythrocytes, effect of methylene blue and other dyes on oxygen consumption (HARROP and BARRON) 207
Blood:
Calcium, inorganic phosphorus, cholesterol, and lipoid phosphorus. I (HARNES) 549
Cell, metabolism. I (HARROP and BARRON) 207
Cells, effect of light. I (EARLE) 457
—, — — —. II (EARLE) 667
—, — — —. III (EARLE) 683
Chemistry, after obstruction of cardiac end of stomach, effect of sodium chloride (HADEN and ORR) 627
—, — temporary obstruction of pylorus (HADEN and ORR) 591
—, in peritonitis (ORR and HADEN) 339
Chlorides, in proteose intoxication (HADEN and ORR) 639

Blood—*continued:*
Corpuscle, white. *See* Leucocyte.
Human, inheritance of agglutinogens demonstrable by immune agglutinins (LANDSTEINER and LEVINE) 731
Serum. *See* Serum.

C

Calcium:
Blood, and blood inorganic phosphorus, cholesterol, and lipoid phosphorus. I (HARNES) 549
Capsule:
Bacillus coli, relation to antibody production (SMITH) 351
Carrier:
Paratyphoid, guinea pig, in endemic stage (NELSON) 647
Cattle:
Bacillus tuberculosis, fate in organs of rabbit (LURIE) 155
Colostrum, cholesterol and cholesterol ester content (SHOPE and GOWEN) 21
Serum, cholesterol and cholesterol ester late in pregnancy and during early lactation (SHOPE and GOWEN) 25
Cell:
Blood, metabolism. I (HARROP and BARRON) 207
— and tissue, effect of light. I (EARLE) 457
— — —, effect of light. II (EARLE) 667
— — —, effect of light. III (EARLE) 683
—, white. *See* Leucocyte.
Cell—*continued:*
Living, conduction of high frequency currents (CHRISTIE) 235
Chemotherapy:
Bartonella bacilliformis infection (NOGUCHI) 619
Chloride(s):
Blood, in proteose intoxication (HADEN and ORR) 639
Cholesterol:
Blood, and blood calcium, inorganic phosphorus, and lipoid phosphorus. I (HARNES) 549
Colostrum, bovine (SHOPE and GOWEN) 21
Serum, of cattle, late in pregnancy and during early lactation (SHOPE and GOWEN) 25
Tuberculosis, influence on, of (SHOPE) 321
Cholesterol ester:
Colostrum, bovine (SHOPE and GOWEN) 21
Serum, of cattle, late in pregnancy and during early lactation (SHOPE and GOWEN) 25
Colostrum:
Cattle, cholesterol and cholesterol ester content (SHOPE and GOWEN) 21
Corpuscle:
Blood, white. *See* Leucocyte.
Crocker Foundation:
Sarcoma 10, malignant fibroblast of (CARREL and EBELING) 105
Cultivation:
Bacillus typhosus, filtrate, local skin reactivity (SHWARTZMAN) 247
Bacterium, production of bacteriophage from cultures (MUCKENFUSS) 723

Cultivation—*continued:*
Fibroblasts, sarcomatous and normal, nitrogen metabolism in pure cultures (BAKER and CARREL) 533

D

Diathermy:
VI (CHRISTIE) 235
Dye:
Oxygen consumption of mammalian and avian erythrocytes, effect on, of (HARROP and BARRON) 207
Dysentery:
Bacillus dysenteriæ, Shiga, toxin production by normal and by phage-resistant strains (MUCKENFUSS and KORB) 277

E

Electricity:
High frequency currents, conduction through living cell (CHRISTIE) 235
Embryo:
Chick, influence of lead on development (HAMMETT and WALLACE) 659
Encephalitis:
Rivers Virus III (RIVERS and STEWART) 603
Virus, herpes, relation of streptococci (OLITSKY and LONG) 199
Endemic:
Paratyphoid, guinea pig, carriers (NELSON) 647
Epidemic:
Puerperal fever, hemolytic streptococci associated with, studied by reciprocal agglutination and absorption of agglutinin tests (MELENEY, ZAYTZEFF, HARVEY, and ZAU) 299
Epinephrine:
Hemolysin formation, effect on, of injection of (MARMORSTON-GOTTESMAN and PERLA) 225
Erythrocyte:
Light, effect *in vitro* (EARLE) 667
Mammalian and avian, effect of methylene blue and other dyes on oxygen consumption (HARROP and BARRON) 207

F

Fever:
Oroya. *See* Oroya fever.
Puerperal. *See* Puerperal fever.
Relapsing. *See* Relapsing fever.
Yellow. *See* Yellow fever.
Fibroblast:
Light, effect *in vitro* (EARLE) 683
Macrophage and, properties. III (CARREL and EBELING) 105
— —, —. IV (CARREL and EBELING) 285
Malignant, of Jensen sarcoma (CARREL and EBELING) 285
—, — Sarcoma 10 of Crocker Foundation (CARREL and EBELING) 105
Sarcomatous and normal, nitrogen metabolism in pure cultures (BAKER and CARREL) 533
Filtration:
Bacillus typhosus culture filtrate, local skin reactivity (SHWARTZMAN) 247
— — toxic filtrate prepared *in vivo*, producing typhoid fever (HARRIS and LARIMORE) 885

Filtration—*continued:*
Sarcoma (Chicken Tumor I—Rous), effect of oxidation of filtrates (MUELLER) 343
Flagellum:
Agglutination, flagellar and somatic (NELSON) 811
Fluid:
Body, changes due to continued loss of external secretion of pancreas (GAMBLE and MCIVER) 859

G

Gastric:
See Stomach.
Gland:
Adrenal. *See* Adrenal.
Growth:
Light environment, especially neon light, influence of, on (BROWN) 31
— —, — neon light, influence of, on hair (BROWN) 57

H

Hair:
Growth, influence of light environment, especially neon light (BROWN) 57
Hemolysin:
Formation, effect of injection of epinephrine (MARMORSTON-GOTTESMAN and PERLA) 225
Hemolytic:
Streptococci, antigenic differences between matt forms and their glossy variants (LANCEFIELD and TODD) 769
—, associated with epidemic of puerperal fever, studied by reciprocal agglutination and absorption of agglutinin tests (MELENEY, ZAYTZEFF, HARVEY, and ZAU) 299
Hemolytic—*continued:*
Streptococci, conversion to non-hemolytic forms (TODD) 493
—, variants, relation to type-specific substance, virulence, and toxin (TODD and LANCEFIELD) 751
Heredity:
Agglutinogens of human blood, inheritance demonstrable by immune agglutinins (LANDSTEINER and LEVINE) 731
d'Herelle phenomenon:
See Twort-d'Herelle phenomenon.
Herpes:
Virus, action of Levaditi strain and of vaccine virus in guinea pig (single and combined effects) (OLITSKY and LONG) 379
— encephalitis and streptococci (OLITSKY and LONG) 199
Hydrolysis:
Bacterial protein, during lysis (HETLER and BRONFENBRENNER) 269

I

Immunity:
Agglutinins, immune, demonstrating inheritance of agglutinogens of human blood (LANDSTEINER and LEVINE) 731
Pneumococcus infection, active and passive immunity induced by immunization with R pneumococci (TILLETT) 791
— vaccine. I (BARACH) 83
— —, active and passive immunity (BARACH) 83

Immunity—*continued:*
Relapsing fever, suppression of first attack with subsequent relapse (MELENEY) 805
Vaccinal, influence on reaction to syphilis (PEARCE) 125
—, and vaccinia, influence on reaction to infection with syphilis (intracutaneous inoculation) (PEARCE) 363
Immunology:
Adrenal. III (MARMORSTON-GOTTESMAN and PERLA) 225
Infection:
Concomitant, reciprocal effects. II (PEARCE) 125
—, — —. III (PEARCE) 363
Intoxication:
Proteose, blood chlorides (HADEN and ORR) 639
In vitro:
Erythrocyte, effect of light (EARLE) 667
Fibroblast, effect of light (EARLE) 683
Leucocyte, effect of light (EARLE) 457
In vivo:
Bacillus typhosus toxic filtrate prepared *in vivo*, producing typhoid fever (HARRIS and LARIMORE) 885
Isomer:
Steric, serological differentiation (LANDSTEINER and VAN DER SCHEER) 315

J

Jaundice:
Obstructive, extracorporeal thrombosis (JOHNSON, SHIONOYA, and ROWNTREE) 871
Jensen:
Sarcoma, malignant fibroblast (CARREL and EBELING) 285

L

Lactation:
Cattle, cholesterol and cholesterol ester in serum during early lactation period (SHOPE and GOWEN) 25
Lead:
Chick embryo development, influence on, of (HAMMETT and WALLACE) 659
Leucocyte:
Light, effect *in vitro* (EARLE) 457
Light:
Blood and tissue cells, effect on, of. I (EARLE) 457
— — — —, effect on, of. II (EARLE) 667
— — — —, effect on, of. III (EARLE) 683
Environment, influence on growth and nutrition (BROWN) 31
—, — — growth of hair (BROWN) 57
—, — — organic constitution (BROWN) 567
Erythrocyte *in vitro*, action on, of (EARLE) 667
Fibroblast *in vitro*, action on, of (EARLE) 683
Leucocyte *in vitro*, action on, of (EARLE) 457
Neon, influence on growth and nutrition (BROWN) 31
—, — — growth of hair (BROWN) 57
—, — — organic constitution (BROWN) 567
Lipoid:
Phosphorus, and calcium, inorganic phosphorus, and cholesterol, in blood. I (HARNES) 549

Lysis:
Hydrolysis of bacterial protein during (HETLER and BRONFENBRENNER) 269

M

Macrophage:
Fibroblast and, properties. III (CARREL and EBELING) 105
— —, —. IV (CARREL and EBELING) 285
Malignancy:
Fibroblast, malignant, of Jensen sarcoma (CARREL and EBELING) 285
—, —, — Sarcoma 10 of Crocker Foundation (CARREL and EBELING) 105
Mammal:
Erythrocytes, effect of methylene blue and other dyes on oxygen consumption (HARROP and BARRON) 207
Metabolism:
Blood cell. I (HARROP and BARRON) 207
Nitrogen, of normal and sarcomatous fibroblasts in pure cultures (BAKER and CARREL) 533
Metal:
Biology. VII (HAMMETT and WALLACE) 659
Methylene blue:
Oxygen consumption of mammalian and avian erythrocytes, effect on, of (HARROP and BARRON) 207
Mosquito:
Yellow fever, incubation period in (BAUER and HUDSON) 147

N

Neon:
Light, influence on growth and nutrition (BROWN) 31
Neon—*continued:*
Light, influence on growth of hair (BROWN) 57
—, — — organic constitution (BROWN) 567
Nitrogen:
Metabolism of normal and sarcomatous fibroblasts in pure cultures (BAKER and CARREL) 533
Nutrition:
Light environment, especially neon light, influence of, on (BROWN) 31

O

Obstruction:
Jaundice, obstructive, extracorporeal thrombosis (JOHNSON, SHIONOYA, and ROWNTREE) 871
Pylorus, temporary, chemical findings in blood (HADEN and ORR) 591
Stomach, cardiac end, effect of sodium chloride on chemical changes in blood (HADEN and ORR) 627
Organ:
Constitution, influence of light environment, especially neon light (BROWN) 567
Oroya fever:
Etiology. XIII (NOGUCHI) 619
Oxidation:
Filtrates of chicken sarcoma (Chicken Tumor I—Rous) (MUELLER) 343
Oxygen:
Consumption of mammalian and avian erythrocytes, effect of methylene blue and other dyes (HARROP and BARRON) 207

P

Pancreas:
Secretion, external, acid-base composition (GAMBLE and McIVER) 849
—, —, body fluid changes due to continued loss (GAMBLE and McIVER) 859
Paratyphoid:
Guinea pig. V (NELSON) 647
Peritonitis:
Blood, chemical changes (ORR and HADEN) 339
Pettit:
Antipoliomyelitic serum, neutralization experiments with poliomyelitis virus (STEWART and HASELBAUER) 449
Phosphorus:
Inorganic, and calcium, cholesterol, and lipoid phosphorus, in blood. I (HARNES) 549
Lipoid, and calcium, inorganic phosphorus, and cholesterol, in blood. I (HARNES) 549
Pneumococcus:
Autolysates, toxic (PARKER and PAPPENHEIMER) 695
Immunity produced with vaccine. I (BARACH) 83
Infection, active and passive immunity induced by immunization with R pneumococci (TILLETT) 791
Intradermal infection (GOODNER) 1, 413
R forms inducing active and passive immunity to pneumococcus infection (TILLETT) 791
Pneumonia:
Experimental, in guinea pigs. I (PARKER and PAPPENHEIMER) 695
Pneumonia—*continued:*
Lobar, mechanism of recovery (SIA, ROBERTSON, and WOO) 513
Poliomyelitis:
Serum, Pettit's antipoliomyelitic, neutralization experiments with poliomyelitis virus (STEWART and HASELBAUER) 449
—, Rosenow's antipoliomyelitic, neutralization experiments with poliomyelitis virus (STEWART and HASELBAUER) 449
Streptococcus, rôle in (LONG, OLITSKY, and STEWART) 431
Precipitin:
Agglutination. II (JONES) 183
Reaction, effect on surface tension of serum (RAMSDELL) 615
Pregnancy:
Cattle, cholesterol and cholesterol ester in serum (SHOPE and GOWEN) 25
Protein:
Bacterial, hydrolysis during lysis (HETLER and BRONFENBRENNER) 269
Proteose:
Intoxication, blood chlorides (HADEN and ORR) 639
Protoplasm:
Amœba dubia, effect of chemical constituents of tubercle bacilli (REZNIKOFF) 193
Puerperal fever:
Hemolytic streptococci associated with epidemic, studied by reciprocal agglutination and absorption of agglutinin tests (MELENEY, ZAYTZEFF, HARVEY, and ZAU) 299

Pylorus:
Obstruction, temporary, chemical findings in blood (HADEN and ORR) 591

R

Reaction:
Precipitin, effect on surface tension of serum (RAMSDELL) 615

Relapsing fever:
Immune phenomenon: suppression of first attack with subsequent relapse (MELENEY) 805
Relapse phenomena (MELENEY) 65

Rheumatism:
Streptococci, indifferent, in throats of rheumatic and non-rheumatic individuals (HITCHCOCK) 403

Rosenow:
Antipoliomyelitic serum, neutralization experiments with poliomyelitis virus (STEWART and HASELBAUER) 449

Rous:
Sarcoma (Chicken Tumor I), effect of oxidation of filtrates (MUELLER) 343

S

Sarcoma:
Crocker Foundation Sarcoma 10, malignant fibroblast of (CARREL and EBELING) 105
Fibroblasts, sarcomatous and normal, in pure cultures, nitrogen metabolism (BAKER and CARREL) 533
Jensen, malignant fibroblast (CARREL and EBELING) 285
Rous Chicken Tumor I, effect of oxidation of filtrates (MUELLER) 343

Secretion:
Gastric, acid-base composition (GAMBLE and MCIVER) 837
Pancreas, external secretion, and bile, acid-base composition (GAMBLE and MCIVER) 849
—, — —, body fluid changes due to continued loss (GAMBLE and MCIVER) 859

Sensitization:
Bacteria, sensitized motile, removal of agglutinin (NELSON) 825

Serology:
Steric isomers, serological differentiation (LANDSTEINER and VAN DER SCHEER) 315
Streptococcus Type I (HITCHCOCK) 393

Serum:
Antibacteriophage, mode of action (MUCKENFUSS) 709
Cattle, cholesterol and cholesterol ester late in pregnancy and during early lactation (SHOPE and GOWEN) 25
Poliomyelitis, Pettit's, neutralization experiments with poliomyelitis virus (STEWART and HASELBAUER) 449
—, Rosenow's, neutralization experiments with poliomyelitis virus (STEWART and HASELBAUER) 449
Surface tension as affected by precipitin reaction (RAMSDELL) 615

Shiga's bacillus:
See Bacillus dysenteriæ.

Skin:
Reactivity, local, to *Bacillus typhosus* culture filtrate (SHWARTZMAN) 247

Sodium chloride:
Obstruction of cardiac end of stomach, effect of sodium chloride on chemical changes in blood (HADEN and ORR) 627

Specificity:
Streptococci, hemolytic, relation of variants to type-specific substance, virulence, and toxin (TODD and LANCEFIELD) 751

Spironema:
recurrentis, relapse phenomena (MELENEY) 65

Stomach:
Obstruction of cardiac end, effect of sodium chloride on chemical findings in blood (HADEN and ORR) 627
Secretions, acid-base composition (GAMBLE and MCIVER) 837

Streptococcus:
Hemolytic, antigenic differences between matt forms and their glossy variants (LANCEFIELD and TODD) 769
—, associated with epidemic of puerperal fever, studied by reciprocal agglutination and absorption of agglutinin tests (MELENEY, ZAYTZEFF, HARVEY, and ZAU) 299
—, conversion to non-hemolytic forms (TODD) 493
—, variants, relation to type-specific substance, virulence, and toxin (TODD and LANCEFIELD) 751
Herpes virus encephalitis and (OLITSKY and LONG) 199
Indifferent. I (HITCHCOCK) 393
—. II (HITCHCOCK) 403

Streptococcus—*continued:*
Indifferent, distribution in throats of rheumatic and non-rheumatic individuals (HITCHCOCK) 403
Poliomyelitis and (LONG, OLITSKY, and STEWART) 431

Suprarenal:
See Adrenal.

Syphilis:
Intracutaneous inoculation, influence of vaccinia and vaccinal immunity on reaction (PEARCE) 363
Vaccinia immunity, influence of, on (PEARCE) 125

T

Tension:
Surface, of serum, as affected by precipitin reaction (RAMSDELL) 615

Throat:
Rheumatic and non-rheumatic individuals, distribution of indifferent streptococci (HITCHCOCK) 403

Thrombosis:
Extracorporeal. VII (JOHNSON, SHIONOYA, and ROWNTREE) 871
—, in obstructive jaundice and after intravenous administration of bile acids (JOHNSON, SHIONOYA, and ROWNTREE) 871

Tissue:
Cells, effect of light. I (EARLE) 457
—, — — —. II (EARLE) 667
—, — — —. III (EARLE) 683

Toxin:
Bacillus typhosus toxic filtrate prepared *in vivo*, producing typhoid fever (HARRIS and LARIMORE) 885
— —, — substances. I (SHWARTZMAN) 247
Pneumococcus toxic autolysates (PARKER and PAPPENHEIMER) 695
Production by normal and by phage-resistant Shiga dysentery bacilli (MUCKENFUSS and KORB) 277
Streptococci, hemolytic, relation of variants to type-specific substance, virulence, and (TODD and LANCEFIELD) 751

Trachoma:
Etiology (NOGUCHI) suppl. 2, pp. 1–53

Tuberculosis:
Bacillus, effect of chemical constituents on protoplasm of *Amœba dubia* (REZNIKOFF) 193
—, human and bovine, fate in organs of rabbit (LURIE) 155
Cholesterol, influence of, on (SHOPE) 321

Tumor: (*See also* Sarcoma.)
Rous, Chicken Tumor I, effect of oxidation of filtrates (MUELLER) 343

Twort-d'Herelle phenomenon:
Bacteriophage. IX (HETLER and BRONFENBRENNER) 269
—. X (MUCKENFUSS and KORB) 277
—. XI (MUCKENFUSS) 709
—. XII (MUCKENFUSS) 723

Typhoid:
Production in guinea pig with *in vivo* prepared toxic filtrate of *Bacillus typhosus* (HARRIS and LARIMORE) 885

V

Vaccine:
Pneumococcus, producing immunity. I (BARACH) 83

Vaccinia:
Immunity, influence on reaction to syphilis (PEARCE) 125
—, and vaccinia, influence on reaction to infection with syphilis (intracutaneous inoculation) (PEARCE) 363
Virus, action in guinea pig, alone and in combination with herpes virus (OLITSKY and LONG) 379

Virulence:
Streptococci, hemolytic, relation of variants to type-specific substance, toxin, and (TODD and LANCEFIELD) 751

Virus:
Herpes, encephalitis, relation of streptococci (OLITSKY and LONG) 199
—, Levaditi strain, action in guinea pig, alone and in combination with vaccine virus (OLITSKY and LONG) 379
Poliomyelitis, neutralization experiments with Pettit's antipoliomyelitic serum (STEWART and HASELBAUER) 449
—, — — — Rosenow's antipoliomyelitic serum (STEWART and HASELBAUER) 449
Rivers Virus III encephalitis (RIVERS and STEWART) 603

Y

Yellow fever:
Incubation period in mosquito (BAUER and HUDSON) 147

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CONTENTS

TODD, E. W., and LANCEFIELD, REBECCA C. Variants of hemolytic streptococci; their relation to type-specific substance, virulence, and toxin. 751

LANCEFIELD, REBECCA C., and TODD, E. W. Antigenic differences between matt hemolytic streptococci and their glossy variants. 769

TILLETT, WILLIAM S. Active and passive immunity to pneumococcus infection induced in rabbits by immunization with R pneumococci. 791

MELENEY, HENRY EDMUND. Suppression of the first attack with subsequent relapse: an immune phenomenon in experimental relapsing fever. 805

NELSON, JOHN B. Observations on flagellar and somatic agglutination. 811

NELSON, JOHN B. The removal of agglutinin from sensitized motile bacteria. 825

GAMBLE, JAMES L., and MCIVER, MONROE A. The acid-base composition of gastric secretions. 837

GAMBLE, JAMES L., and MCIVER, MONROE A. Acid-base composition of pancreatic juice and bile. 849

GAMBLE, JAMES L., and MCIVER, MONROE A. Body fluid changes due to continued loss of the external secretion of the pancreas. 859

JOHNSON, WALTER R., SHIONOYA, TAKUJI, and ROWNTREE, LEONARD G. Studies in experimental extracorporeal thrombosis. VII. Extracorporeal thrombosis in experimental obstructive jaundice and after the intravenous administration of bile acids. Plate 20. 871

HARRIS, WILLIAM H., and LARIMORE, OGILVIE M. The production of experimental typhoid fever in the guinea pig with an *in vivo* prepared toxic filtrate of *Bacillus typhosus*. Plates 21 to 23. 885

INDEX TO VOLUME XLVIII. 897

SUPPLEMENT No. 2.

AUGUST 1, 1928.

NOGUCHI, HIDEYO. The etiology of trachoma. Plates 1–31.

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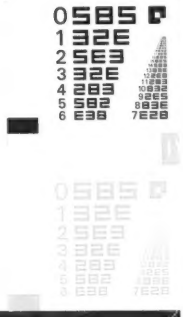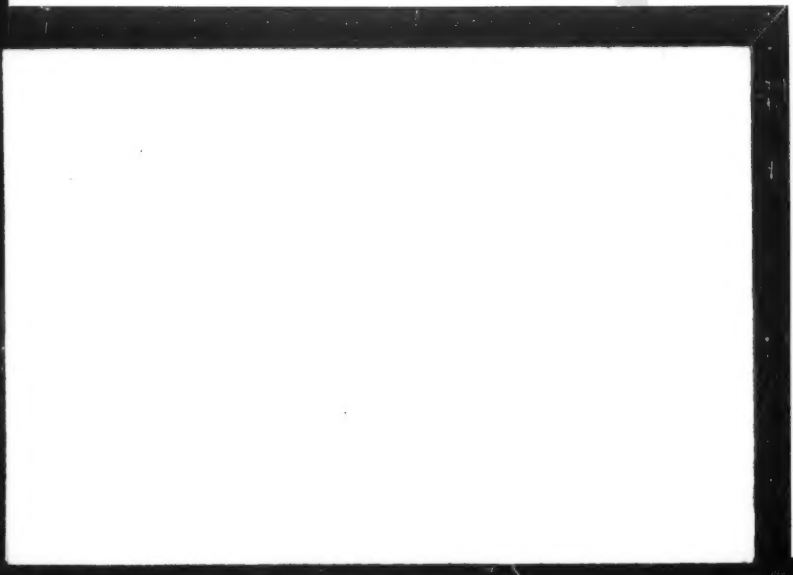
C37